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(71) Applicants and
(72) Inventors: GHADIRI, M., Reza [US/US]; 5133 Seagrove Court, San Diego, CA 92130 (US). KIM, Hui-Sun [US/US]; 1321 Everett Street, El Cerrito, CA 94530 (US). FERNANDEZ-LOPEZ, Sara [US/US]; 3945 Alma Street, Palo Alto, CA 94306 (US). WILCOXEN, Keith [US/US]; 730 Pennsylvania Avenue, San Diego, CA 92103 (US).

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(54) Title: ANTI-MICROBIAL PEPTIDES AND COMPOSITIONS

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(57) Abstract: The present invention provides novel anti-microbial agents and compositions that include cyclic peptides having an amino sequence of alternating D-and L-α-amino acids. Alternatively, the cyclic peptides are made from β-amino acids.

ANTI-MICROBIAL PEPTIDES AND COMPOSITIONS

This application claims priority from provisional application U.S. Ser. No. 60/288,990, filed May 4, 2001. Work relating to this application was

5   supported by a grant from the National Institutes of Health (GM 52190). The government may have certain rights in the invention.

FIELD OF THE INVENTION

The present invention provides cyclic peptide anti-microbials. Anti-

10   microbial peptides and compositions of the invention can quickly kill microbes causing an infection without causing substantial or undesired harm to mammalian cells. The cyclic peptides of the invention may be composed of a sequence of α-amino acids that have a repeating D, L-backbone chirality, or homochiral β-amino acids.

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BACKGROUND OF THE INVENTION

Antibiotic resistance is gaining in importance as a medical problem because more microorganisms are becoming resistant to greater numbers of antibiotics. *See, Antibiotic Resistance, A Growing Threat*, website at

20   fda.gov/oc/opacom/hottopics/anti_resist.html; P.J. Koplan et al., *Preventing emerging infections diseases. A Strategy for the 21ˢᵗ Century*, U.S. Department of Health & Human Services, Center for Disease Control & Prevention, Atlantic, GA (1998). For example, some strains of *Staphylococcus aureus* have developed resistance to many distinct antibiotics and are now described as

25   having a "multi-resistant" phenotype. Unfortunately, due to extensive use of antibiotics, such multi-resistant strains of microorganisms are now found in the general population and within hospitals. Hospital-acquired bacterial infections occur in 5% of patients admitted to the hospital (about 2 million patients per year in the United States). Such infections cause an estimated 20,000 deaths per

30   year, and contribute to an additional 60,000 hospital deaths every year. It is estimated that such infections cause about 7.5 million added days of hospitalization and cost an extra $1 billion dollars in health care costs each year.

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"New" antibiotics are often structurally related to, or structurally derived from, a previous generation of antibiotics. For example, cephalosporin is structurally related to penicillin. While these structural analogs of known antibiotics can be successful for a time, increasing reports of resistance often

5   develop as the "new" antibiotic becomes widely used. The progression of β-lactam antibiotics illustrates this problem. Since the introduction of penicillin in the 1940's, microbes have evolved resistance to β-lactam antibiotics, typically by acquired or enhanced catabolism of these drugs through the enzyme β-lactamase that is found in many bacterial strains. While researchers have made

10   successive changes to the basic β-lactam antibiotic structure in order to generate new antibiotics that are more resistant to degradation by β-lactamases, microorganisms have responded by producing different types or larger amounts of β-lactamases.

Approximately seventy percent of hospital-acquired bacterial infections

15   are resistant to at least one of the most commonly prescribed antibiotics. Such resistance often develops because commonly used antibiotics act comparatively slowly on the metabolism or cellular structure of the bacteria. For example, a common mechanism by which β-lactam antibiotic-resistance develops is via plasmid amplification of an encoded protein that can breakdown the β-lactam

20   antibiotic. When β-lactam antibiotics are routinely prescribed, as often occurs in hospitals, a small segment of the bacterial population that is resistant to such antibiotics can propagate, the plasmids encoding the resistance function can amplify, and the highly resistant strain of bacteria can spread throughout the hospital.

25   This problem has been addressed by the production of new generations of antibiotics. For example, valinomycin is a cyclic depsipeptide with an alternating D-D-L-L chiral motif that employs ester linkages within the ring structure. However, the activity, selectivity, *in vivo* stability, toxicity and bioavailability of valinomycin and other such peptides are not optimal.

30   Accordingly, there is a need for fast-acting, non-toxic anti-microbial agents to combat microbial infections, especially infections that are resistant to currently available antibiotics, which are easily and cheaply manufactured.

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SUMMARY OF THE INVENTION

The present invention provides new, fast-acting anti-microbial cyclic peptides and compositions for treating and/or preventing microbial infections. The present anti-microbial agents are highly effective for many microbial

5   species, including multiply-resistant bacteria as well as lethal methicillin-resistant and vancomycin-resistant microbial strains. Cyclic peptides are fast-acting, proteolytically stable and easy to synthesize. In many embodiments, substantially no microbes can be detected within just a few hours of exposure to the cyclic peptides of the invention. Still others do not cause substantial

10   undesired lysis of mammalian cells, for example, as measured by hemolysis of erythrocytes.

The invention provides anti-microbial cyclic peptides and pharmaceutical compositions thereof for human and veterinary applications wherein the cyclic peptides comprise a sequence of from four to about sixteen alternating D- and L-

15   α-amino acids, and wherein the cyclic peptide has preferential cytotoxic activity against the target microbial organism and does not have substantial undesired activity against mammalian cells. Activity against mammalian cells can be measured, for example, by the ability of peptides to cause hemolysis of mammalian red blood cells. Such cyclic peptides and pharmaceutical

20   compositions can be used for treating or preventing a microbial infection in a mammal. Unlike many anti-microbial agents, preferred cyclic peptides of the invention have been shown to be bactericidal, and not merely bacteristatic at minimum inhibitory concentrations.

The cyclic peptides of the invention generally have a minimum inhibitory

25   concentration at which substantially no cells of at least one target microbe grow *in vitro* that is less than one half the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells. In other embodiments, the minimum inhibitory concentration can be less than one quarter to less than one fifth the peptide concentration needed to cause 50% hemolysis of mammalian red blood

30   cells. In other embodiments, the minimum inhibitory concentration is less than at least one tenth to less than at least one twentieth the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells.

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A large variety of microbial infections can be treated with the cyclic peptides of the invention. For example, the target microbial organism can be a bacterial strain, a yeast strain, a fungal strain, a single-cell organism, a single-cell parasite or a related organism. In one embodiment, the target microbial

5   organisms are gram-positive bacteria or gram-negative bacteria.

The cyclic peptides of the invention are believed to self-assemble into supramolecular structures within or by association with microbial membranes. Such supramolecular structures can be, for example, nanotubes, barrels or groups of associated, axially parallel nanotubes, carpets of associated nanotubes, or

10   mixtures thereof. These types of supramolecular structures can induce microbial cell membrane permeation, depolarization or disruption (e.g., lysis) selectively over mammalian cell membrane permeation, depolarization or disruption.

Cyclic peptides of the invention may have a plurality of amino acids having side chains with affinity for biomolecules integral to microbial cell

15   membranes. Such biomolecules can facilitate selective assembly of the cyclic peptides into supramolecular structures on or within microbial membranes. Most mammalian cell membranes generally do not have the same biomolecular composition as most microbial membranes. Cyclic peptides of the invention will not preferably associate with mammalian cell membrane over microbial cell

20   membranes at desired anti-microbial concentrations.

The cyclic peptides can have a half-life in the bloodstream of the mammal of about six hours or less, at an amount that is effective against microbial infections.

Such an effective amount is an amount of the cyclic peptide that is

25   sufficient to induce death or lysis of microbial cells in single or divided doses without inducing an undesired amount of death or lysis of mammalian cells. Preferred cyclic peptides of the invention induce substantially no hemolysis of red blood cells at anti-microbially effective doses.

In some embodiments, the cyclic peptides of the invention can be

30   administered in a single dosage and yet are effective for treating microbial infections. Alternatively, the cyclic peptides of the invention can be administered continuously or in multiple doses over a period of one to ten days. Effective amounts of the present cyclic peptides include about 0.1 mg/kg to

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about 100 mg/kg of body weight, alternatively about 0.5 mg/kg to about 50

mg/kg of body weight, about 1.0 mg/kg to about 30 mg/kg of body weight, and

other amounts set forth herein.

The cyclic peptides of the invention generally have about 25% to about

5   88% D- and/or L-polar amino acids. In some embodiments, the percentage of

polar amino acids can be from about 33% or 50% to about 65% or 88% of the

total number of D- and L-amino acids. Thus, for example, an eight residue cyclic

peptide of the invention can have at least one, alternatively, two to seven polar

D- and L-amino acids. Other eight residue cyclic peptides will have three to five

10   polar D- and L-amino acids for example. In some embodiments, for example, six

residue cyclic peptides of the invention can have two to five polar D- and L-

amino acids. Other six residue cyclic peptides may have three to four polar D-

and L-amino acids. At least one of these polar D- or L-amino acids may be

adjacent to at least one other polar D- or L-amino acid. Alternatively, at least one

15   polar D- or L-amino acid may be adjacent only to nonpolar D- or L-amino acids.

A variety of polar amino acids are available to one of skill in the art.

Examples of polar D- and L-α-amino acids that can be utilized in the peptides of

the invention include the D- or L-enantiomers of serine, threonine, asparagine,

glutamine, aspartic acid, cysteine, homocysteine, glutamic acid, histidine,

20   arginine, lysine, hydroxylysine or ornithine.

The cyclic peptides of the invention generally have about 25% to about

88% D- and/or L-ionizable amino acids. In some embodiments, the percentage

of ionizable amino acids can be from about 33% or 50% to about 65% or 88% of

the total number of D- and L-amino acids. Thus, for example, a six or eight

25   residue cyclic peptide can have at least one, or alternatively two or three or more

ionizable D- and/or L-amino acids. In other embodiments, the cyclic peptides of

the invention can have four to six ionizable D- and/or L-amino acids. Such an

ionizable D- or L-amino acid can be adjacent to at least one other polar or

ionizable D- or L-amino acid. Alternatively, the cyclic peptides of the invention

30   can have at least one ionizable D- or L-amino acid that is adjacent only to
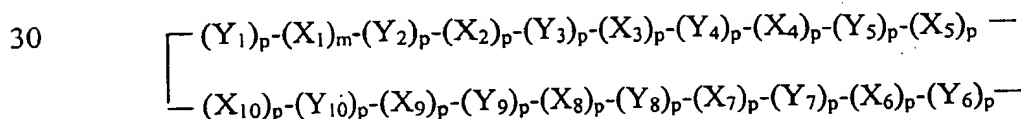
nonpolar D- or L-amino acids.

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Many types of ionizable amino acids are available to one of skill in the art and the invention contemplates all such ionizable amino acids. Examples of ionizable D- and/or L-amino acids include the D- or L-enantiomers of arginine, aspartic acid, glutamic acid, histidine, lysine, hydroxylysine or ornithine.

5   The cyclic peptides of the invention can have nonpolar D- and/or L-amino acid residues. The cyclic peptides of the invention generally have about 12% to about 75% D- and/or L-nonpolar amino acids. In some embodiments, the percentage of nonpolar amino acids can be from about 50% to about 67% or 75% of the total number of D- and/or L-amino acids. Thus, for example, an eight

10   residue cyclic peptide of the invention can have at least one, alternatively, two to seven nonpolar D- and/or L-amino acids. Other eight residue cyclic peptides may have three to five nonpolar D- and/or L-amino acids. In some embodiments, for example, six residue cyclic peptides of the invention have two to five nonpolar D- and/or L-amino acids. Other six residue cyclic peptides may have three to

15   four nonpolar D- and/or L-amino acids. At least one of these nonpolar D- and/or L-amino acids may be adjacent to at least one other nonpolar D- and/or L-amino acid. Alternatively, at least one nonpolar D- or L-amino acid may be adjacent only to polar D- or L-amino acids.

Many types of nonpolar amino acids are available to one of skill in the

20   art and the invention contemplates all such nonpolar amino acids. Examples of nonpolar amino acids include the D- and L-enantiomers of alanine, valine, isoleucine, leucine, methionine, norleucine, phenylalanine, tyrosine or tryptophan.

In addition to the amino acids found naturally in proteins, other naturally

25   occurring amino acids may be used, as well as non-naturally occurring and synthetic amino acids.

In one embodiment, the cyclic peptides of the invention can have an amino acid sequence having formula I:

30

$$\begin{bmatrix} (Y_1)_p\text{-}(X_1)_m\text{-}(Y_2)_p\text{-}(X_2)_p\text{-}(Y_3)_p\text{-}(X_3)_p\text{-}(Y_4)_p\text{-}(X_4)_p\text{-}(Y_5)_p\text{-}(X_5)_p \\ (X_{10})_p\text{-}(Y_{10})_p\text{-}(X_9)_p\text{-}(Y_9)_p\text{-}(X_8)_p\text{-}(Y_8)_p\text{-}(X_7)_p\text{-}(Y_7)_p\text{-}(X_6)_p\text{-}(Y_6)_p \end{bmatrix}$$

I

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wherein:

m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a

5   polar D- or L-α-amino acid; and

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately

nonpolar D- or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

10   In another embodiment, the cyclic peptides of the invention can have an

amino acid sequence having formula II:

$$\left[\begin{array}{c} \text{(D-X}_1\text{-L-X}_2\text{)}_m - \text{(D-Y}_1\text{-L-Y}_2\text{)}_p - \text{(L-X}_3\text{-D-X}_4\text{)}_p - \text{(L-Y}_3\text{-D-Y}_4\text{)}_p \\ \\ \text{(L-Y}_8\text{-D-Y}_7\text{)}_p - \text{(D-X}_8\text{-L-X}_7\text{)}_p - \text{(D-Y}_6\text{-L-Y}_5\text{)}_p - \text{(L-X}_6\text{-D-X}_5\text{)}_p \end{array} \right]$$
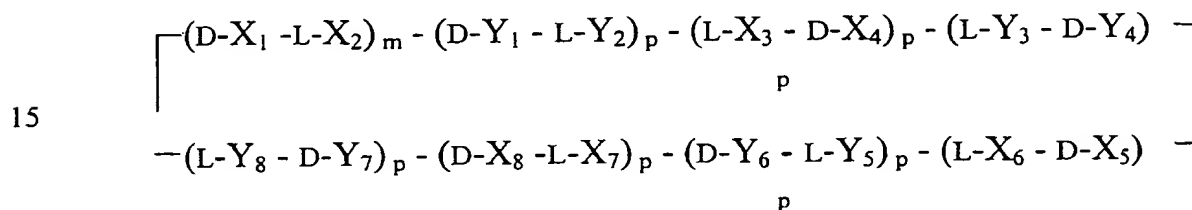
II

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wherein:

m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, and X_8 is separately a polar D- or

25   L-α-amino acid;

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, and Y_8 is separately nonpolar D-

or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

30   In yet another embodiment, the cyclic peptides of the invention can have

an amino acid sequence having formula III:

$$\left[\begin{array}{c} \text{(X}_1\text{)}_p\text{-(X}_2\text{)}_p\text{-(X}_3\text{)}_p\text{-(X}_4\text{)}_m\text{-(X}_5\text{)}_p\text{-(X}_6\text{)}_p\text{-(X}_7\text{)}_p\text{-(X}_8\text{)}_p\text{-(X}_9\text{)}_p\text{-(X}_{10}\text{)}_p \\ \text{(Y}_{10}\text{)}_p\text{-(Y}_9\text{)}_p\text{-(Y}_8\text{)}_p\text{-(Y}_7\text{)}_p\text{-(Y}_6\text{)}_p\text{-(Y}_5\text{)}_p\text{-(Y}_4\text{)}_p\text{-(Y}_3\text{)}_p\text{-(Y}_2\text{)}_p\text{-(Y}_1\text{)}_p \end{array} \right]$$

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III

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wherein:

    m is an integer ranging from 1 to 7;

    each p is separately an integer ranging from 0 to 7;

    each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a polar D- or L-α-amino acid;

    each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately nonpolar D- or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about sixteen alternating D- and L-α amino acids.

In yet another embodiment, the cyclic peptide has an amino acid sequence of formula IVa or IVb:

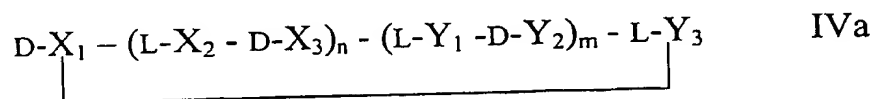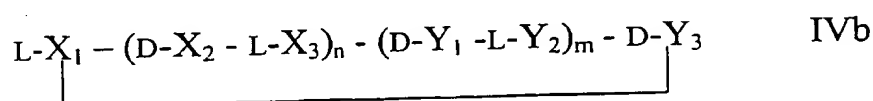$$\text{D-}X_1 - (\text{L-}X_2 - \text{D-}X_3)_n - (\text{L-}Y_1 - \text{D-}Y_2)_m - \text{L-}Y_3 \qquad \text{IVa}$$

$$\text{L-}X_1 - (\text{D-}X_2 - \text{L-}X_3)_n - (\text{D-}Y_1 - \text{L-}Y_2)_m - \text{D-}Y_3 \qquad \text{IVb}$$

wherein:

    n is an integer ranging from 0 to 4;

    m is an integer ranging from 1 to 7;

    X_1, X_2 and X_3 are each separately a polar amino acid;

    Y_1, Y_2 and Y_3 are each separately nonpolar amino acid; and

wherein the cyclic peptide has an even number of from four to about sixteen alternating D- and L-α amino acids.

In another embodiment, the cyclic peptide has an amino acid sequence of formula Va or Va:

$$\text{D-}X_1 - \text{L-}X_2 - (\text{D-}Y_1 - \text{L-}Y_2)_q \qquad\qquad \text{L-}X_1 - \text{D-}X_2 - (\text{L-}Y_1 - \text{D-}Y_2)_q$$
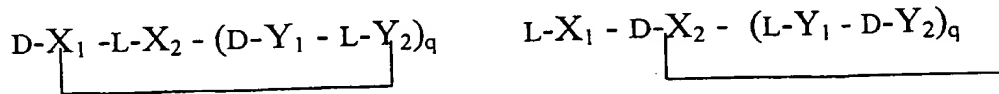
$$\textbf{Va} \qquad\qquad\qquad\qquad\qquad\qquad \textbf{Vb}$$

wherein:

q is an integer ranging from 2 to 7;

X_1 and X_2 are separately polar amino acids;

Y_1 and Y_2 are separately nonpolar amino acids.

5   The claimed cyclic peptides of the above formulae, however, exclude those composed entirely of nonpolar amino acids. Moreover, a peptide having any one of the following sequences may be excluded from one or more of the above formulae:

cyclo-[-(Gln- D-Ala)$_4$-]

10   cyclo-[-(Gln- D-Leu)$_4$-]

cyclo-[-(Gln- D-Val)$_4$-]

cyclo-[-(Phe- D-Leu)$_4$-]

cyclo-[-(Phe- D-Ala)$_4$-]

cyclo-[-(Gln- D-Ala-Glu- D-Ala)$_2$-]

15   cyclo-[-(Gln- D-Phe-Glu- D-Phe)$_2$-]

cyclo-[-(Gln- D-Ala-Glu- D-Ala)$_3$-]

cyclo-[-(Trp- D-Leu)$_3$-Gln- D-Leu-]

cyclo-[-Gln- (D-Leu-Trp)$_4$- D-Leu-]

cyclo-[-Lys-D-Leu-Lys-D-Leu-Lys-D-Leu-Lys-D-Leu];

20   cyclo-[-Arg- D-Leu -Arg- D-Leu- Arg- D-Leu-Arg- D-Leu-];

cyclo-[-Glu- D-Leu -Glu- D-Leu-Glu- D-Leu-Glu- D-Leu-].

Additionally, the following sequences may or may not be excluded from the above formulae and associated compositions:

cyclo-[-D-Arg-Gln-D-Arg-Ala- D-Trp-Leu- D-Trp-Trp-];

25   cyclo-[-D-Arg-Gln-D-Arg-Leu-D-Trp-Leu- D-Trp-Trp-];

cyclo-[-D-Arg-Gln-D-Arg-Val-D-Trp-Leu- D-Trp-Trp-];

cyclo-[-D-Arg-Gln-D-Arg-Phe-D-Trp-Leu- D-Trp-Trp-]; or

cyclo-[-D-Arg-Gln-D-Arg-Trp-D-Trp-Leu- D-Trp-Trp-].

Cyclic peptides having any of the above sequences, however, including any of

30   the excluded sequences, may be included within or excluded from pharmaceutical compositions and peptides of the present invention.

Cyclic peptides often have, for example, about four to about sixteen D- and L-α-amino acids. In other embodiments, the cyclic peptides have about six
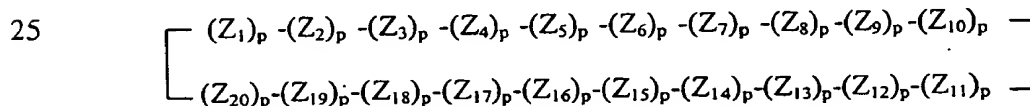
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to about ten or twelve D- and L-α-amino acids. In still other embodiments, cyclic peptides of about six or eight D- and L-α-amino acids are employed.

The pharmaceutical compositions of the invention can include an effective amount of at least one of the cyclic peptides of the invention, or two or more different cyclic peptides of the invention. These compositions also include a pharmaceutically effective carrier.

According to the invention, the cyclic peptides need not be made from D- or L-α-amino acids and can alternatively have a sequence of from three to about ten homochiral β-amino acids. Such β-amino acids are available to one of skill in the art. Beta amino acids can be substituted at the α- or β-carbons by one to two substituents. Mono-substituted beta amino acids of either S or R chirality can be employed for the construction of cyclic β-peptides, provided that the cyclic β-peptide is homochiral. Disubstituted β-amino acids employed in the homochiral β-peptides of the present invention have the relative R,R or S,S diastereomeric configuration, provided that the cyclic β-peptide is homochiral. In other words, the β-amino acids of the cyclic β-peptides of the present invention should be homochiral, i.e., the substituents at the α and/or β backbone carbons should be all S and/or S,S, or all R and/or R,R. Cyclic peptides having β-amino acids generally have at least one β-amino acid with at least one polar side chain. Preferred β-peptides cause substantially no undesirable lysis of mammalian cells.

In one embodiment, the cyclic β-peptides of the invention can have an amino acid sequence of formula VI:

$$\left[\begin{array}{l} (Z_1)_p - (Z_2)_p - (Z_3)_p - (Z_4)_p - (Z_5)_p - (Z_6)_p - (Z_7)_p - (Z_8)_p - (Z_9)_p - (Z_{10})_p \\ (Z_{20})_p - (Z_{19})_p - (Z_{18})_p - (Z_{17})_p - (Z_{16})_p - (Z_{15})_p - (Z_{14})_p - (Z_{13})_p - (Z_{12})_p - (Z_{11})_p \end{array} \right]$$

VI

wherein:

each p is separately an integer ranging from 0 to 7;

each $Z_1, Z_3, Z_5, Z_7, Z_9, Z_{11}, Z_{13}, Z_{15}, Z_{17},$ and Z_{19} is separately a monosubstituted β-amino acid;

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each Z_2, Z_4, Z_6, Z_8, Z_{10}, Z_{12}, Z_{14}, Z_{16}, Z_{18}, and Z_{20} is separately a disubstituted β-amino acid; and

wherein the cyclic β-peptide has a sequence of from three to about ten homochiral β-amino acids. The invention therefore also contemplates

5   pharmaceutical compositions that include an effective amount of at least one cyclic peptide, or two or more different cyclic peptides, wherein these cyclic peptide(s) have a sequence of from three to about ten homochiral β-amino acids, for example, as provided by formula VI.

The invention also provides a method of treating a microbial infection in

10   humans and other animals, which comprises contacting microbial cells with a cyclic peptide comprising a sequence of from four to about sixteen amino acids, wherein the sequence has alternating D- and L-α-amino acids, in an amount sufficient to induce microbial cell death without inducing an undesirable amount of mammalian or animal cell death. Such cyclic peptides can alternatively have

15   a sequence of from three to about ten homochiral β-amino acids.

The invention further provides a method of identifying cyclic peptides selectively cytotoxic to a target cell-type comprising screening one or more cyclic peptides for induction of cell death in target cells without induction of substantial or undesired cell death in a second cell type. Such cyclic peptides

20   may comprise, for example, an alternating D- and L- α amino acid sequence of between four and about sixteen amino acids. Alternatively, such cyclic peptides may have a sequence of from three to about ten homochiral β-amino acids.

The invention also provides a method of identifying cyclic peptides selectively cytotoxic to a target cell-type comprising: (a) making a

25   combinatorial library of cyclic peptides, wherein each cyclic peptide in the combinatorial library comprises an alternating D- and L- α amino acid sequence of between four and about sixteen amino acids; and (b) screening cyclic peptides from the combinatorial library for induction of cell death in target cells without induction of substantial or undesired cell death in a second cell type.

30   Alternatively, such cyclic peptides may have a sequence of from three to about ten homochiral β-amino acids. The library can be used to generate single cyclic peptides or mixtures of cyclic peptides. Mixtures of cyclic peptides that show

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anti-microbial activity can then be further screened to identify one or more anti-microbially active cyclic peptides in one or more of the mixtures, which can then be isolated or synthesized and re-tested for induction of cell death in target cells without induction of substantial or undesired cell death in a second cell type.

5   The invention further provides a method of identifying cyclic peptides selectively cytotoxic to a target cell-type. This method involves the step of rationally designing at least one cyclic peptide comprising an alternating D- and L- α amino acid sequence of between four and sixteen amino acids. Alternatively, such cyclic peptides may have a sequence of from three to about

10   ten homochiral β-amino acids. The method further involves screening such rationally designed cyclic peptides for induction of cell death in target cells without induction of substantial or undesired cell death in a second cell type.

         Rationally designing a cyclic peptide can involve identifying at least one effective cyclic peptide from a combinatorial library that can induce cell death in

15   target cells without inducing substantial or undesired cell death in a second cell type and exchanging at least one amino acid for a different amino acid in the alternating D- and L- α amino acid sequence of the effective cyclic peptide to generate a rationally designed cyclic peptide. The rationally designed cyclic peptide can then be screened for induction of cell death in target cells without

20   inducing substantial or undesired cell death in a second cell type. Alternatively, such cyclic peptides may have a sequence of from three to about ten homochiral β-amino acids.

         In these types of screening methods, the target cell-type can be a microbe and the second cell type can be a mammalian cell, for example, a mammalian

25   red blood cell. Cell death of the second cell type can be detected by detecting hemolysis of red blood cells.

         The above methods can further include screening a third cell type. Such screening can include determining whether a peptide induces substantial or undesired cell death in the third cell type. The method can also include

30   determining the minimum inhibitory dose at which a peptide can kill substantially all, or inhibit the growth, of the target cells.

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Screening can be performed *in vitro* by separately contacting a peptide with the target cell type and with other cell types (e.g., a second or third or other cell type). Alternatively, screening can be performed *in vivo* by administering at least one peptide to a test animal comprising the target cell type and another cell

5 type or types and determining whether the peptide is toxic to the target cell type but does not have substantial or undesired toxicity to another cell type or types. Such a method can also include determining whether the peptide adversely affects the health of the animal. Determining whether the peptide adversely affects the health of the animal can include examining the bodily fluids or the

10 anatomy of the animal by pathological or histological methods.

The invention further provides a method of identifying cyclic peptides capable of selective association with one or more target biomolecules on a selected cell surface, comprising contacting a solution of cyclic peptides, each peptide comprising between four to about sixteen amino acids in an alternating

15 D- and L-α amino acid sequence, or a peptide comprising from three to about ten β-amino acids, with the target biomolecule(s) and determining, for example, whether the peptides spontaneously assemble into a supramolecular structure that selectively associates with the biomolecule(s). The target biomolecule can be displayed, for example, on the surface of a living cell or on the surface of a

20 liposome. Alternatively, the peptide can be contacted with the target biomolecule(s). The method can further include determining the structure of the peptides that spontaneously assemble into the supramolecular structure that selectively associates with the biomolecule(s).

The present invention also provides methods of evaluating or confirming

25 therapeutically effective dosages for treating a microbial infection with a cyclic peptide having an amino acid sequence of alternating D- and L-α-amino acids, or a cyclic peptide comprising from three to about ten β-amino acids, that includes determining the minimum inhibitory concentration of the cyclic peptide at which substantially no bacteria grow *in vitro*.

30 The present invention also provides a composition comprising one or more of the present cyclic peptides with one or more other anti-microbial agents.

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Such agents include penicillin, vancomycin, erythromycin and other therapeutic agents.

Mammals and birds may be treated by the methods and compositions described and claimed herein. Such mammals and birds include humans, dogs,

5   cats, and livestock, for example, horses, cattle, sheep, goats, chickens, turkeys and the like.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 provides a schematic drawing of the self-assembly of the present

10   cyclic peptides into nanotubules. An eight-residue cyclic D, L-α-peptide with alternating D- and L-amino acids is depicted to the left, with emphasis on its flat ring-shaped conformation. Side chains (R) decorate the outside surface of the cyclic peptide. Upon self-assembly, a series of cyclic peptides align and are believed to undergo intermolecular hydrogen bonding to form a tubular structure

15   referred to herein as a nanotube (center). Self-assembly is believed to be directed by inter-subunit backbone-backbone hydrogen bonding resulting in a β-sheet-like open-ended hollow tubular supramolecular structure. This β-sheet like hydrogen bonding pattern is shown to the right. For clarity most side chains are omitted.

20   Figure 2 illustrates the modes of membrane permeation that are accessible to peptide supramolecular structures. Depending on the composition and sequence of amino acids employed in the cyclic peptides, supramolecular structures that may be formed can interact with the membranes of cells through, for example, supramolecular assembly of (a) pores, (b) barrel stave structures, or

25   (c) carpet-like modes of action. Cyclic peptides are depicted as ring structures.

Figure 3 graphically illustrates the rate of membrane depolarization of intact S. aureus (ATCC 25923) upon exposure to cyclic peptide SEQ ID NO:8 (open circles), cyclic peptide SEQ ID NO:18 (open triangles), and cyclic peptide SEQ ID NO:26 (open squares). Membrane depolarization was monitored by the

30   change in the fluorescence emission intensity of the membrane potential-sensitive dye diSC3. The excitation wavelength (λ_{ex}) was 622 nm and the emission wavelength (λ_{em}) was 670 nm. Peptides were added at t = 1 min and

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aliquots taken at t = 4 min to obtain viable bacteria counts. In all cases at least a 1000 fold reduction in viability upon the addition of the peptides was observed.

    Figure 4 provides a plot of the apparent proton transport (Figure 4a) and the carboxyfluoroscein release (Figure 4b) mediated by peptide SEQ ID NO:11

5   (cyclo-[Gln-D-Lys-(Trp-D-Leu)$_2$-Trp-D-Lys-]), as expressed in fractional fluorescence changes as a function time. For Figure 4b, the peptide was added at about 100 seconds, and the detergent triton X-100 was added at about 200 seconds.

    Figure 5 provides an attenuated total reflectance (ATR) infrared (IR)

10   spectra for analyzing the orientation of peptide SEQ ID NO:11 (cyclo-[-Gln-D-Lys-(Trp-D-Leu)$_2$-Trp-D-Lys-] in DMPC multibilayers. The solid trace indicates absorbance of parallel-polarized light; the dashed trace provides absorbance of perpendicular-polarized light.

    Figure 6 provides a dose-effect curve illustrating the *in vivo* efficacy of

15   three cyclic peptides as anti-microbial compounds. As illustrated, the survival rate of mice challenged with lethal doses of methicillin-resistant *Staphylococcus aureus* is dramatically improved as the dosage of the cyclic peptide is increased. Three different cyclic peptides were tested: (a) peptide SEQ ID NO:18 (PD$_{50}$ =5±2 mg/kg), (b) peptide SEQ ID NO:17 (PD$_{50}$=6±2 mg/kg), and (c) peptide

20   SEQ ID NO:26 (PD$_{50}$=10±2 mg/kg). PD50 is the protective dose where 50% of animals survive.

    Figure 7 provides a structural comparison of supramolecular structures composed of: (a) cyclic β-tetrapeptides, and (b) cyclic D, L-α-octapeptides. This figure illustrates that, due to the unidirectional arrangement of the polar

25   backbone amide groups, cyclic β-tetrapeptide supramolecular structures may possess a macrodipole moment reminiscent of an α-helix, while cyclic D, L-α-octapeptide supramolecular structures will, under most circumstances, not have such a net dipole moment. For clarity most side chains are omitted from the nanotube structures depicted in Figure 7a and 7b.

30   Figure 8a provides a "time-killing curve" illustrating the number of surviving *S. aureus* (MRSA, ATCC 33591) in log cfu/ml as a function of time after exposure to one of the following peptides at MIC concentrations:

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cyclo[KHLWLW] (closed circles), cyclo[KRKWLWLW] (closed triangles) or cyclo[KQRWLWLW] (open squares), where the underlining indicates that the amino acid is a D-amino acid.

Figure 8b provides a "time-killing curve" illustrating the number of surviving *E. coli* JM109 (DE3) in log cfu/ml as a function of time after exposure to one of the following peptides at the MIC concentration: cyclo[KRKWLWLW] (closed triangles), cyclo[KQRWLWLW] (closed circles), cyclo[KHLWLW] (closed squares) or cyclo[KKLWLW] (open squares), where the underlining indicates that the amino acid is a D-amino acid.

Figure 9 provides a "time-killing curve" illustrating the number of surviving *S. aureus* (MRSA) (ATCC 33592)(log cfu/ml) as a function of time after exposure to the cyclo[KSKWLWLW] peptide at 37 °C, where the underlining indicates that the amino acid is a D-amino acid. The effects of several concentrations of the cyclo[KSKWLWLW] peptide on separate cultures are shown.

Figure 10 provides a "time-killing curve" illustrating the number of surviving *S. aureus* (MRSA) (ATCC 33592)(log cfu/ml) as a function of time after exposure of the cyclo[KSKWLWLW] peptide at room temperature, where the underlining indicates that the amino acid is a D-amino acid. The effects of several concentrations of the cyclo[KSKWLWLW] peptide on separate cultures are shown.

Figure 11 provides a "time-killing curve" illustrating the number of surviving *Eneterococus faecium* SP180 (cfu/ml) as a function of time after exposure to the cyclo[KSKWLWLW] peptide at 37 °C, where the underlining indicates that the amino acid is a D-amino acid. *E. faecium* SP180 is vancomycin resistant (vanA) and multiply resistant to several FDA approved antibiotics. The effects of several concentrations of the cyclo[KSKWLWLW] peptide on separate cultures are shown.

Figure 12 provides a "time-killing curve" illustrating the number of surviving *Eneterococus faecium* SP180 (cfu/ml) as a function of time after exposure to the cyclo[KSKWLWLW] peptide at room temperature, where the underlining indicates that the amino acid is a D-amino acid. *E. faecium* SP180 is

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vancomycin resistant (vanA) and multiply resistant to several FDA approved antibiotics. The effects of several concentrations of the cyclo[KSKWLWLW] peptide on separate cultures are shown.

Figure 13 provides a "time-killing curve" illustrating the number of

5   surviving *S. aureus* (MRSA) (ATCC 33592)(cfu/ml) as a function of time after exposure to the cyclo[RRKWLWLW] peptide at 37 °C, where the underlining indicates that the amino acid is a D-amino acid. The effects of several concentrations of the cyclo[RRKWLWLW] peptide on separate cultures are shown.

10   Figure 14 provides a "time killing curve" illustrating the number of surviving *S. aureus* (MRSA) (ATCC 33592)(cfu/ml) as a function of time after exposure to the cyclo[RRKWLWLW] peptide at room temperature, where the underlining indicates that the amino acid is a D-amino acid. The effects of several concentrations of the cyclo[RRKWLWLW] peptide on separate cultures

15   are shown.

Figure 15 provides a "time-killing curve" illustrating the number of surviving *Eneterococus faecium* SP180 (cfu/ml) as a function of time after exposure to the cyclo[RRKWLWLW] peptide at 37 °C, where the underlining indicates that the amino acid is a D-amino acid. *E. faecium* SP180 is

20   vancomycin resistant (vanA) and multiply resistant to several FDA approved antibiotics. The effects of several concentrations of the cyclo[RRKWLWLW] peptide on separate cultures are shown.

Figure 16 provides a "time-killing curve" illustrating the number of surviving *Eneterococus faecium* SP180 (cfu/ml) as a function of time after

25   exposure to the cyclo[RRKWLWLW] peptide at room temperature, where the underlining indicates that the amino acid is a D-amino acid. *E. faecium* SP180 is vancomycin resistant (vanA) and multiply resistant to several FDA approved antibiotics. The effects of several concentrations of the cyclo[RRKWLWLW] peptide on separate cultures are shown.

30   Figure 17 provides a "time-killing curve" illustrating the number of surviving *S. aureus* (MRSA) (ATCC 33592)(cfu/ml) as a function of time after

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cells were exposed to penicillin G at 37 °C. The effects of several concentrations penicillin G on separate cultures are shown.

Figure 18 provides a "time-killing curve" illustrating the number of surviving *S. aureus* (MRSA) (ATCC 33592)(cfu/ml) as a function of time after

5   cells were exposed to penicillin G at room temperature. The effects of several concentrations of penicillin G on separate cultures are shown.

Figure 19 provides a "time-killing curve" illustrating the number of surviving *Eneterococus faecium* SP180 (cfu/ml) as a function of time after cells were exposed to penicillin G at 37 °C. *E. faecium* SP180 is vancomycin

10   resistant (vanA) and multiply resistant to several FDA approved antibiotics. The effects of several concentrations penicillin G on separate cultures are shown.

Figure 20 provides a "time-killing curve" illustrating the number of surviving *Eneterococus faecium* SP180 (cfu/ml) as a function of time after cells were exposed to penicillin at room temperature. *E. faecium* SP180 is

15   vancomycin resistant (vanA) and multiply resistant to several FDA approved antibiotics. The effects of several concentrations of penicillin on separate cultures are shown.

Figure 21 provides a thin section electron microscopy image of untreated *S. aureus* (ATCC 25923) displaying a normal intact membrane.

20   Figure 22 provides a thin section electron microscopy image of *S. aureus* (ATCC 25923) after exposure to 2XMIC concentration of cyclo[KSKWLWLW] for 2 h. The image provides direct visualization of the membrane mode of action. Arrows denote abnormal membrane structures caused by the peptide action.

25   Figure 23 provides a thin section electron microscopy image of *S. aureus* (ATCC 25923) after exposure to 2xMIC concentration of cyclo[KSKWLWLW] for 2 h. The image provides direct visualization of the membrane mode of action. Arrows denote abnormal membrane structures caused by the peptide action.

30   Figure 24 illustrates the protective effects of the present cyclic peptides on survival of mice lethally infected with *S. aureus* MRSA (ATCC 33591). The mouse peritonitis model was used with intraperitoneal infection (right side) of

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bacteria in 5 % mucin and peptide injection (IP left side) 45-60 min post

infection. Survival was monitored over 14 days (death as an end point); eight

mice were tested per dose. In Figure 24a, the mice were injected with varying

concentrations of cyclo[RRKWLWLW]HCl. In Figure 24b, the mice were

5   injected with varying concentrations of cyclo[KQRWLWLW]HCl.

Figure 25 illustrates the protective effects of the present cyclic peptides

on survival of mice infected with *S. aureus* MRSA (ATCC 33591). The mouse

peritonitis model was used with intraperitoneal infection (right side) of bacteria

in 5 % mucin and peptide injection (IP left side) 45-60 min post infection.

10   Survival was monitored over 14 days (death as an end point); eight mice were

tested per dose. In Figure 25a, the mice were injected with varying

concentrations of cyclo[KSKWLWLW]HCl. In Figure 25b, the mice were

injected with varying concentrations of cyclo[KKKWLWLW]HCl.

Figure 26 illustrates the protective effects of the present cyclic peptides

15   on survival of mice infected with *S. aureus* MRSA (ATCC 33591). The mouse

peritonitis model was used with intraperitoneal infection (right side) of bacteria

in 5 % mucin and peptide injection (IP left side) 45-60 min post infection.

Survival was monitored over 14 days (death as an end point); eight mice were

tested per dose. The mice were injected with varying concentrations of

20   cyclo[KKLWLW]HCl.

Figure 27 illustrates the protective effects of the present cyclic peptides

on survival of mice infected with vancomycin resistant *E. faecium*

(VREF)(ATCC 51575). The mouse peritonitis model was used with

intraperitoneal infection (right side) of bacteria in 5 % mucin and peptide

25   injection (IP left side) 45-60 min post infection. Survival was monitored over 14

days (death as an end point); eight mice were tested per dose. In Figure 27a, the

mice were injected with varying concentrations of cyclo[RRKWLWLW]HCl. In

Figure 27b, the mice were injected with varying concentrations of

cyclo[KSKWLWLW]HCl.

30   Figure 28 illustrates the protective effects of the present cyclic peptides

on survival of mice infected with *S. aureus* MRSA (ATCC 33591). The mouse

peritonitis model was used with intraperitoneal infection (right side) of bacteria

in 5 % mucin and peptide injection (IP left side) 45-60 min post infection.

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Survival was monitored over 14 days (death as an end point); four mice were tested per dose. In Figure 28a, the mice were injected subcutaneously (upper neck compartment) with varying concentrations of cyclo[RRKWLWLW]HCl. In Figure 28b, the mice were injected intravenously with varying concentrations

5   of cyclo[RRKWLWLW]HCl. Peptide doses indicated are for each injection. Five doses at the indicated mg/kg quantities were administered intravenously 8-12 h apart.

Figure 29 illustrates the toxicity of peptides administered via intraperitoneal route. In Figure 29a, the mice were injected with varying

10   concentrations of cyclo[RRKWLWLW]HCl. In Figure 29b, the mice were injected with varying concentrations of cyclo[KQRWLWLW]HCl. Mice were monitored over a period of 14 days for activity and mortality. Four mice per dose were used in each experiment.

Figure 30 illustrates the toxicity of peptides administered via

15   intraperitoneal route. In Figure 30a, the mice were injected with varying concentrations of cyclo[KSKWLWLW]HCl. In Figure 30b, the mice were injected with varying concentrations of cyclo[KKLWLW]HCl. Mice were monitored over a period of 14 days for activity and mortality. Four mice per dose were used in each experiment.

20   **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides small cyclic peptides and compositions that quickly and selectively kill microbes without substantial or undesired toxicity toward mammalian cells. The present invention includes cyclic peptides, and pharmaceutical compositions comprising cyclic peptides, with

25   either a sequence of alternating D-, and L-α-amino acids, or a sequence of β-amino acids, that can sample flat, ring-shaped conformations. Such ring-shaped conformations project the amino acid side chains of the cyclic peptides away from the center of the ring and orient the amide backbone approximately perpendicular to the plane of the ring structure. It is believed that under

30   conditions that favor hydrogen bonding, such as side chain charge neutualization through interactions with cell membrane constituents and/or contact with low dielectric constant environments of cell membranes, the cyclic peptides can self-assemble via intermolecular hydrogen bonding to form supramolecular

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structures. Cyclic peptides that simply contain one or more D-amino acids do not adopt a flat ring-shaped conformation and do not have the backbone conformation needed for self-assembly of the cyclic peptide into supramolecular structures. Target microbial organisms against which the present cyclic peptides

5   are effective include microbes, including any single cell organism or parasite that has a cellular membrane and that can infect a mammal. For example, target microbial organisms include bacteria, fungi, helminths, protozoa, yeast strains and other single cell organisms. Cyclic peptides of the invention have been found to be active against both gram-negative and gram-positive bacteria.

10   Small differences in environment, for example, the difference in composition of different cellular membranes, can influence the course and nature of the proposed assembly process. This feature is used in the present invention both to target and to optimize the anti-microbial activity of selected cyclic peptides against particular microbial species, while providing substantially no

15   toxicity, or no undesired toxicity, in mammalian cells at therapeutically effective doses and dose regimens. Supramolecular structures are believed to respond to their immediate environment through dynamic self-assembling/disassembling processes to quickly find the most thermodynamically favored assembly. It is believed that, during assembly, peptide supramolecular structures sense and

20   respond to the environment of a cellular membrane by sampling various topologically related assemblies. In non-target mammalian membranes at therapeutically desirable concentrations, it is believed that the preferred cyclic peptides of the invention do not or cannot adopt a thermodynamically favorable supramolecular structure. Thus, mammalian membranes are not substantially or

25   undesirably affected by the presence of such cyclic peptides. However, in selected microbial membranes the present cyclic peptides are believed to form unique energetically favorable supramolecular structures that destabilize (e.g., lyse), permeablize and/or depolarize the microbial membrane, thereby disrupting microbial transmembrane ion and electrical gradients and other vital functions,

30   and quickly leading to microbial cell death.

Small changes in amino acid sequence of a cyclic peptide can be amplified into large differences at the supramolecular level. Thus, changes in the structure of a cyclic peptide may constrain peptide interaction and limit

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formation of supramolecular structures to particular cellular membranes that have particular membrane constituents, membrane partitioning properties, uptake properties, and the like.

Another feature of the present self-assembling peptide supramolecular structures is believed to be the potential for a given cyclic peptide to form a number of diastereomeric nanotube assemblies. This property stems from the fact that backbone-backbone hydrogen bonding are believed primarily to direct the self-assembly of the nanotube structure. Differently stacked subunits can give rise to topoisomeric supramolecular structures that share the same or nearly the same tubular β-sheet-like hydrogen bonded backbone structure. The variety of supramolecular structures assembled from a single cyclic peptide minimizes the probability that microbes can develop resistance to these antibiotic agents.

By varying the peptide sequence while retaining a cyclic D- and L-α-peptide backbone, or the cylic β-peptide backbone, a multitude of cyclic peptides can quickly be screened or evaluated for the ability to selectively target and assemble in microbial membranes. They can also be screened and evaluated for anti-microbial activity by testing for increased membrane permeability, depolarization, or destabilization.

The term "amino acid," includes the residues of the natural α-amino acids (e.g. Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Hyl, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) in D or L form, as well as β-amino acids, synthetic and unnatural amino acids. Many types of amino acid residues are useful in the cyclic peptides and the invention is not limited to natural, genetically-encoded amino acids. Examples of amino acids that can be utilized in the cyclic peptide described herein can be found, for example, in Fasman, 1989, CRC Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Inc., and the references cited therein. Another source of a wide variety of amino acid residues is provided by the website of RSP Amino Acids Analogues, Inc. (www.amino-acids.com).

The term "mammal," as used herein, refers to an animal, in general, a warm-blooded animal, which is susceptible to or has a microbial infection. Mammals include cattle, buffalo, sheep, goats, pigs, horses, dogs, cats, rats, rabbits, mice, and humans. Also included are other livestock, domesticated

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animals and captive animals. The term "farm animals" includes chickens, turkeys, fish, and other farmed animals.

As used herein, a "nanotube" or "nanotubule" is a small tubule that may spontaneously form from the cyclic peptides of the present invention. The

5   present cyclic peptides are believed to stack to form supramolecular structures composed of nanotubes. Hydrogen bonding between cyclic peptide is believed to help drive the self-assembly of the supramolecular structures from the cyclic peptide. Each nanotube has a pore in the center of the tube that is surrounded by the series of peptide backbones of the stacked cyclic peptides that form the

10   nanotubes. The size of the pore depends upon the number of amino acids in the cyclic peptides that form the nanotube. In general, depending on the ring size of the cyclic peptides employed, ions, sugars, and other small molecules can travel through the pores of the nanotubes. Larger molecules can also flow through pores formed from larger cyclic peptides and supramolecular structures formed

15   of aggregates of nanotubes. For example, in some embodiments the supramolecular structure is thought to be a barrel-like structure composed of clusters of nanotubes. In other embodiments, the supramolecular structure is thought to be a "carpet" or "carpet-like" arrangement of nanotubes.

The term "peptide" as used herein includes a sequence of from four to

20   sixteen amino acids residues in which the α-carboxyl group of one amino acid is joined by an amide bond to the main chain (α- or β-) amino group of the adjacent amino acid. The peptides provided herein for use in the described and claimed methods and compositions are cyclic. Peptide sequences specifically recited herein are written with the amino terminus on the left and the carboxy

25   terminus on the right. However, where the peptides are shown in cyclic form, the first amino acid in the sequence is arbitrarily chosen. Moreover, for formulae of cyclic peptides where the sequence extends onto two lines, the sequence on the second line extends from the N-terminal side on the right to the C-terminal side on the left.

30   According to the present invention, "supramolecular structures" are multi-subunit structures, e.g. nanotubes, barrels and carpets of nantubules, which are believed to be formed through "noncovalent" assembly of cyclic peptides.

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Supramolecular structures may be contrasted with molecular or polymeric systems in which the product of covalent bond formation between reactants or monomers. The proposed peptide supramolecular structures are thermodynamically controlled assemblies that can undergo reversible structural

5   assembly and disassembly. Such assembly-disassembly will depend, for example, on the environment, subunit structure, side group selection, side group interaction, and the nature and combination of noncovalent forces operating on the system. In contrast, covalent polymeric structures have been used to design kinetically stable structures rather than structures that assemble and dissemble in

10   response to the environment. Hence, one attractive feature of the present compositions containing peptides that can form supramolecular structures is their ability to select amongst various cell membrane types. Such selection is driven by favorable thermodynamic forces determined by the composition of the cyclic peptide relative to the cell membrane environment and the molecular and/or

15   supramolecular constituents of the cell membrane.

       The term "substantially no" with reference to self-assembly, hemolysis, toxicity or cellular lysis, or the like, means that little or no self-assembly, hemolysis, toxicity, cellular lysis or the like is present at the tested or desired peptide dosage or concentration. By way of example, "substantially no"

20   hemolysis can mean that less than about 20%, alternatively less than 15% or less than 10%, or no detectable, hemolysis at the tested or desired peptide dosage or concentration has occurred. Similarly, "substantially no" toxicity or lysis can mean that less than about 20%, alternatively less than 15% or less than 10%, or no detectable, toxicity or lysis at the tested or desired peptide dosage or

25   concentration has occurred. In other embodiments, "substantially no" hemolysis, toxicity or lysis means that less than about 5%, or no detectable, hemolysis, toxicity or lysis, etc., at the tested or desired peptide dosage or concentration has occurred.

       The term "therapeutically effective amount" is that amount sufficient to

30   control a microbial infection. A therapeutically effective amount generally controls the amount of microbes and/or a disease state characterized by the presence of microbes in the infected mammal, for example, by at least about 20%, by at least about 40%, by at least about 60%, or by at least about 80%

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relative to untreated subjects. In some embodiments, a therapeutically effective
amount controls the amount of microbes and/or a disease state characterized by
the presence of microbes in the infected mammal by at least about 90% or more.
These percentages refer to a decrease in the amount of microbes found in the

5　infected mammal and/or the decrease in symptoms associated with a disease
state characterized by the presence of microbes in the infected mammal relative
to untreated subjects. In other embodiments, a "therapeutically effective
amount" is that amount of cyclic peptide needed to permeabilize, depolarize or
destabilize the cellular membrane of the target microbial organism causing the

10　infection. The term "therapeutically effective amount" can also be the amount
needed to kill the target microbial organisms causing the infection. An effective
amount of the therapeutic agent necessary to control microbes can vary
according to factors such as the type of microbes, the amount of microbes
already present in the animal, the age, sex, health and weight of the mammal,

15　and the ability of the cyclic peptides of the present invention to control microbial
infections in the mammal.

Therapeutically effective amounts of the peptide and peptide
compositions can also be used to prevent microbial infection, including
preventing a recurrence of microbial infection.

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Peptides, Peptide Variants, and Derivatives Thereof

The present invention provides cyclic peptides and compositions
including cyclic peptides that have an amino acid sequence of alternating D- and
L-amino acids that is between four to about sixteen, alternatively about six to

25　about sixteen amino acids in length. Alternatively, the cyclic peptides of the
present invention can have between three to about ten β-amino acids. In
general, the cyclic D, L-α-peptides do not include the amino acids proline and
glycine. According to the invention, β-amino acids can be substituted at the α-
or β-carbons, or both. Mono-substituted β-amino acids of either S or R chirality

30　can be employed for the construction of cyclic β-peptides, provided that the
cyclic beta peptide is homochiral. Disubstituted β-amino acids employed in the
present invention must have the relative R,R or S,S diastereomeric configuration,

provided that the β-amino acid residues in a cyclic peptide structure are homochiral. Cyclic peptides having β-amino acids generally have at least one β-amino acid with at least one polar side chain.

5   The cyclic peptides of the present invention are believed to undergo self-assembly to form supramolecular structures that, upon assembly in or on a microbial membrane, can cause depolarization and/or permeablization and/or destailization of the microbial membrane. In some cases, the cyclic peptides cause lysis of the microbe, e.g. a bacterium. Self-assembly into supramolecular structures is thought to occur by stacking of the cyclic peptides in an anti-parallel

10   fashion or a parallel fashion with formation of β-sheet hydrogen bonds between adjacent cyclic peptides. However, it is believed that the preferred cyclic peptides do not readily self-assemble into supramolecular structures in mammalian cellular membranes as measured, for example, in an assay for toxicity in mammalian cells or hemolysis of mammalian red blood cells at tested

15   or therapeutically effective doses.

        Cyclic peptides of the present invention can be made from α-amino acids or β-amino acids. The amino acid sequence of the present cyclic peptides includes at least one polar amino acid in the case of D,L α-amino acid cyclic peptides, or at least one polar side chain in the case of cyclic β-peptides. The

20   percentage of polar amino acids can range, for example, from about 25% or 33% to about 65% or 88%. However, in some embodiments a majority of the amino acids are polar. For example, the percentage of polar amino acids can be from about 50% to about 88% of the total number of amino acids. The exact number of polar and nonpolar amino acids depends on the size and the properties sought

25   for a given cyclic peptide. In some embodiments, the sizes for the present cyclic peptides are about six to about ten D,L α-amino acids or three to about ten β-amino acids. In other embodiments, the size for the present cyclic peptides is about six to about eight D,L α-amino acids or four to about six β-amino acids. Thus, for example, an eight residue cyclic peptide of the invention can have at

30   least one, alternatively, two to seven polar D- and/or L-α-amino acids. Other eight residue cyclic peptides will have three to five polar D- and/or L-α-amino acids for example. Preferred eight residue cyclic peptides have three, four or

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five polar amino acids. In some embodiments, for example, six residue cyclic peptides of the invention can have two to five polar D- and/or L-α-amino acids. Other six residue cyclic peptides may have three to four polar D- and/or L-α-amino acids. At least one of these polar D- or L-α-amino acids may be adjacent

5   to at least one other polar D- or L-α-amino acid. Alternatively, at least one polar D- or L-α-amino acid may be adjacent only to nonpolar D- or L-α-amino acids. Beta peptides having about four to about eight β-amino acids may have, for example, about two to twelve polar side chains, depending on the level of α and β backbone substitution.

10   The cyclic D- L-α-peptides of the invention generally have about 25% to about 88% ionizable amino acid residues. In some embodiments, the percentage of ionizable amino acids can be from about 33% or 50% to about 65% or 88% of the total number of D- and/or L-amino acids. Thus, for example, a six or eight residue cyclic peptide can have at least one, or alternatively two or three or more

15   ionizable D- and/or L-amino acids. In other embodiments, the cyclic peptides of the invention can have four to six ionizable D- and/or L-amino acids. Such an ionizable D- or L-amino acid can be adjacent to at least one other polar or ionizable D- or L-amino acid. Alternatively, the cyclic peptides of the invention can have at least one ionizable D- or L-amino acid that is adjacent only to

20   nonpolar D- or L-amino acids. The cyclic β-peptides of the invention generally have about 25% to about 88% ionizable amino acid side chains. In some embodiments, the percentage of ionizable amino acid side chains can be from about 33% or 50% to about 65% or 88% of the total number of amino acid side chains. Thus, for example, a four to six residue cyclic β-peptide can have at

25   least one, or alternatively two or three or more ionizable amino acid side chains. In other embodiments, the cyclic β-peptides of the invention can have four to six ionizable amino acid side chains.

        The cyclic peptides of the invention can have nonpolar D- and/or L-amino acid residues. The number of non-polar amino acids chosen can vary as the size

30   of the peptide varies and as the selected microbial membrane environment varies. The cyclic peptides of the invention generally have about 12% to about 75% D- and L-nonpolar amino acids. In some embodiments, the percentage of

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nonpolar amino acids can be from about 50% to about 67% or 75% of the total number of D- and L-amino acids. Thus, for example, an eight residue cyclic peptide of the invention can have at least one, alternatively, two to seven nonpolar D- and/or L-amino acids. Other eight residue cyclic peptides may have

5   three to five nonpolar D- and/or L-amino acids. In some embodiments, for example, six residue cyclic peptides of the invention have two to five nonpolar D- and/or L-amino acids. Other six residue cyclic peptides may have three to four nonpolar D- and/or L-amino acids. At least one of these nonpolar D- or L-amino acids may be adjacent to at least one other nonpolar D- or L-amino acid.

10   Alternatively, at least one nonpolar D- or L-amino acid may be adjacent only to polar D- or L-amino acids. In general, the cyclic peptides do not include the amino acid proline or glycine, but certain cyclic peptides may have good activity even though proline or glycine is included.

According to the invention, β-amino acids can have non-polar side chains

15   at the α- or β-carbons, or both. The number of non-polar amino acid side chains chosen can vary as the size of the peptide varies and as the selected microbial membrane environment varies. The cyclic β-peptides of the invention generally have about 12% to about 75% nonpolar amino acid side chains. In some embodiments, the percentage of nonpolar amino acid side chains can be from

20   about 50% to about 67% or 75% of the total number of amino acid side chains. Thus, for example, an eight residue cyclic β-peptide of the invention can have at least one, alternatively, two to seven nonpolar amino acid side chains. Other eight residue cyclic β-peptides may have three to five nonpolar amino acid side chains. In some embodiments, for example, six residue cyclic β-peptides of the

25   invention have two to five nonpolar amino acid side chains. Other six residue cyclic β-peptides may have three to four nonpolar amino acid side chains.

Amino acids used in the cyclic peptides can be genetically encoded amino acids, naturally occurring non-genetically encoded amino acids, or synthetic amino acids. Both L- and D-enantiomers of any of the above are

30   utilized in the cyclic peptides. The amino acid notations used herein for the twenty genetically encoded L-amino acids and some examples of non-encoded amino acids are provided in Table 1.

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Table 1

Amino Acid	One-Letter Symbol	Common Abbreviation
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val
B-Alanine		Bala
2,3-Diaminopropionic acid		Dpr
A-Aminoisobutyric acid		Aib
N-Methylglycine (sarcosine)		MeGly
Ornithine		Orn
Citrulline		Cit
t-Butylalanine		t-BuA
t-Butylglycine		t-BuG
N-methylisoleucine		MeIle
Phenylglycine		Phg

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Amino Acid	One-Letter Symbol	Common Abbreviation
Cyclohexylalanine		Cha
Norleucine		Nle
Naphthylalanine		Nal
Pyridylalanine		
3-Benzothienyl alanine		
4-Chlorophenylalanine		Phe(4-Cl)
2-Fluorophenylalanine		Phe(2-F)
3-Fluorophenylalanine		Phe(3-F)
4-Fluorophenylalanine		Phe(4-F)
Penicillamine		Pen
1,2,3,4-Tetrahydro-isoquinoline-3-carboxylic acid		Tic
B-2-thienylalanine		Thi
Methionine sulfoxide		MSO
Homoarginine		Harg
N-acetyl lysine		AcLys
2,4-Diamino butyric acid		Dbu
P-Aminophenylalanine		Phe(pNH$_2$)
N-methylvaline		MeVal
Homocysteine		Hcys
Homoserine		Hser
E-Amino hexanoic acid		Aha
Δ-Amino valeric acid		Ava
2,3-Diaminobutyric acid		Dab

Certain commonly encountered amino acids that are not genetically
encoded and that can be present in the cyclic peptides of the invention include,
but are not limited to, β-alanine (b-Ala) and other omega-amino acids such as 3-
aminopropionic acid (Dap), 2,3-diaminopropionic acid (Dpr), 4-aminobutyric
acid and so forth; α-aminoisobutyric acid (Aib); ε-aminohexanoic acid (Aha); δ-
aminovaleric acid (Ava); methylglycine (MeGly); ornithine (Orn); citrulline
(Cit); t-butylalanine (t-BuA); t-butylglycine (t-BuG); N-methylisoleucine

30

(MeIle); phenylglycine (Phg); cyclohexylalanine (Cha); norleucine (Nle); 2-naphthylalanine (2-Nal); 4-chlorophenylalanine (Phe(4-Cl)); 2-fluorophenylalanine (Phe(2-F)); 3-fluorophenylalanine (Phe(3-F)); 4-fluorophenylalanine (Phe(4-F)); penicillamine (Pen); 1,2,3,4-

5   tetrahydroisoquinoline-3-carboxylic acid (Tic); .beta.-2-thienylalanine (Thi); methionine sulfoxide (MSO); homoarginine (hArg); N-acetyl lysine (AcLys); 2,3-diaminobutyric acid (Dab); 2,3-diaminobutyric acid (Dbu); p-aminophenylalanine (Phe(pNH$_2$)); N-methyl valine (MeVal); homocysteine (hCys) and homoserine (hSer).   Additional amino acid analogs contemplated

10 include phosphoserine, phosphothreonine, phosphotyrosine, hydroxyproline, gamma-carboxyglutamate, hippuric acid, octahydroindole-2-carboxylic acid, statine, α-methyl-alanine, para-benzoyl-phenylalanine, propargylglycine, and sarcosine. Peptides that are encompassed within the scope of the invention can have any of foregoing amino acids in the L- or D- configuration, or any other

15 amino acid known to one of skill in the art.

        Amino acids that are substitutable for each other generally reside within similar classes or subclasses. As known to one of skill in the art, amino acids can be placed into different classes depending primarily upon the chemical and physical properties of the amino acid side chain. For example, some amino acids

20 are generally considered to be hydrophilic or polar amino acids and others are considered to be hydrophobic or nonpolar amino acids. Polar amino acids include amino acids having acidic, basic or hydrophilic side chains and nonpolar amino acids include amino acids having aromatic or hydrophobic side chains.   Nonpolar amino acids may be further subdivided to include, among others,

25 aliphatic amino acids. The definitions of the classes of amino acids as used herein are as follows:

        "Nonpolar Amino Acid" refers to an amino acid having a side chain that is uncharged at physiological pH, that is not polar and that is generally repelled by aqueous solution. Examples of genetically encoded hydrophobic amino acids

30 include Ala, Ile, Leu, Met, Trp, Tyr and Val. Examples of non-genetically encoded nonpolar amino acids include t-BuA, Cha and Nle.

        "Aromatic Amino Acid" refers to a nonpolar amino acid having a side chain containing at least one ring having a conjugated π-electron system

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(aromatic group). The aromatic group may be further substituted with substituent groups such as alkyl, alkenyl, alkynyl, hydroxyl, sulfonyl, nitro and amino groups, as well as others. Examples of genetically encoded aromatic amino acids include phenylalanine, tyrosine and tryptophan. Commonly

5   encountered non-genetically encoded aromatic amino acids include phenylglycine, 2-naphthylalanine, β-2-thienylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, 4-chlorophenylalanine, 2-fluorophenylalanine, 3-fluorophenylalanine and 4-fluorophenylalanine.

"Aliphatic Amino Acid" refers to a nonpolar amino acid having a

10   saturated or unsaturated straight chain, branched or cyclic hydrocarbon side chain. Examples of genetically encoded aliphatic amino acids include Ala, Leu, Val and Ile. Examples of non-encoded aliphatic amino acids include Nle.

"Polar Amino Acid" refers to a hydrophilic amino acid having a side chain that is charged or uncharged at physiological pH and that has a bond in

15   which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Polar amino acids are generally hydrophilic, meaning that they have an amino acid having a side chain that is attracted by aqueous solution. Examples of genetically encoded polar amino acids include asparagine, cysteine, glutamine, lysine and serine. Examples of non-genetically encoded polar amino

20   acids include citrulline, homocysteine, N-acetyl lysine and methionine sulfoxide.

"Acidic Amino Acid" refers to a hydrophilic amino acid having a side chain pK value of less than 7. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Examples of genetically encoded acidic amino acids include aspartic acid (aspartate) and

25   glutamic acid (glutamate).

"Basic Amino Acid" refers to a hydrophilic amino acid having a side chain pK value of greater than 7. Basic amino acids typically have positively charged side chains at physiological pH due to association with hydronium ion. Examples of genetically encoded basic amino acids include arginine, lysine and

30   histidine. Examples of non-genetically encoded basic amino acids include the non-cyclic amino acids ornithine, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid and homoarginine.

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"Ionizable Amino Acid" refers to an amino acid that can be charged at a physiological pH. Such ionizable amino acids include acidic and basic amino acids, for example, D-aspartic acid, D-glutamic acid, D-histidine, D-arginine, D-lysine, D-hydroxylysine, D-ornithine, L-aspartic acid, L-glutamic acid, L-

5   histidine, L-arginine, L-lysine, L-hydroxylysine or L-ornithine.

As will be appreciated by those having skill in the art, the above classifications are not absolute. Several amino acids exhibit more than one characteristic property, and can therefore be included in more than one category. For example, tyrosine has both a nonpolar aromatic ring and a polar hydroxyl

10   group. Thus, tyrosine has several characteristics that could be described as nonpolar, aromatic and polar. However, the nonpolar ring is dominant and so tyrosine is generally considered to be nonpolar. Similarly, in addition to being able to form disulfide linkages, cysteine also has nonpolar character. Thus, while not strictly classified as a hydrophobic or nonpolar amino acid, in many

15   instances cysteine can be used to confer hydrophobicity or nonpolarity to a peptide.

The classifications of the above-described genetically encoded and non-encoded amino acids are summarized in Table 2, below. It is to be understood that Table 2 is for illustrative purposes only and does not purport to be an

20   exhaustive list of amino acid residues that may comprise the peptides and peptide analogues described herein. Other amino acid residues that are useful for making the peptides described herein can be found, e.g., in Fasman, 1989, CRC Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Inc., and the references cited therein. Another source of amino acid residues is

25   provided by the website of RSP Amino Acids Analogues, Inc. (www.amino-acids.com). Amino acids not specifically mentioned herein can be conveniently classified into the above-described categories on the basis of known behavior and/or their characteristic chemical and/or physical properties as compared with amino acids specifically identified.

30

TABLE 2

Classification	Genetically Encoded	Genetically Non-Encoded
Nonpolar		

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Classification	Genetically Encoded	Genetically Non-Encoded
Aromatic	F, Y, W	Phg, Nal, Thi, Tic, Phe(4-Cl), Phe(2-F), Phe(3-F), Phe(4-F), Pyridyl Ala, Benzothienyl Ala
Aliphatic	A, V, L, I	t-BuA, t-BuG, MeIle, Nle, MeVal, Cha, bAla, MeGly, Aib
Other Nonpolar	M, G, P	
Polar	.	
Acidic	D, E	
Basic	H, K, R	Dpr, Orn, hArg, Phe(p-NH$_2$), DBU, A$_2$ BU
Neutral Polar	S, T, Y, Q, N, D, E, H, R, K, C	Cit, AcLys, MSO, hSer, Orn, Hcys
Cysteine-Like	C	Pen, hCys, β-methyl Cys

In some embodiments, polar amino acids contemplated by the present invention include, for example, arginine, asparagine, aspartic acid, cysteine,

5   glutamic acid, glutamine, histidine, homocysteine, lysine, hydroxylysine, ornithine, serine, threonine, the corresponding β-amino acids, and structurally related amino acids. In one embodiment the polar amino is an ionizable amino acid such as arginine, aspartic acid, glutamic acid, histidine, hydroxylysine, lysine, or ornithine.

10   Examples of nonpolar or nonpolar amino acid residues that can be utilized include, for example, alanine, valine, leucine, methionine, isoleucine, phenylalanine, tryptophan, tyrosine and the like.

In addition, the amino acid sequence of a peptide can be modified so as to result in a peptide variant that includes the substitution of at least one amino

15   acid residue in the peptide for another amino acid residue, including substitutions that utilize the D rather than L form.
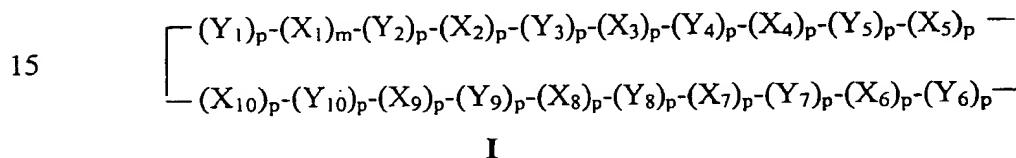
One or more of the residues of the peptide can be exchanged for another, to alter, enhance or preserve the biological activity of the peptide. Such a variant can have, for example, at least about 10% of the biological activity of the

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corresponding non-variant peptide. Conservative amino acid substitutions are often utilized, i.e., substitutions of amino acids with similar chemical and physical properties, as described above.

Hence, for example, conservative amino acids substitutions involve
5   exchanging aspartic acid for glutamic acid; exchanging lysine for arginine or histidine; exchanging one nonpolar amino acid (alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, valine,) for another; and exchanging one polar amino acid (aspartic acid, asparagine, glutamic acid, glutamine, glycine, serine, threonine, etc.) for another. After the substitutions
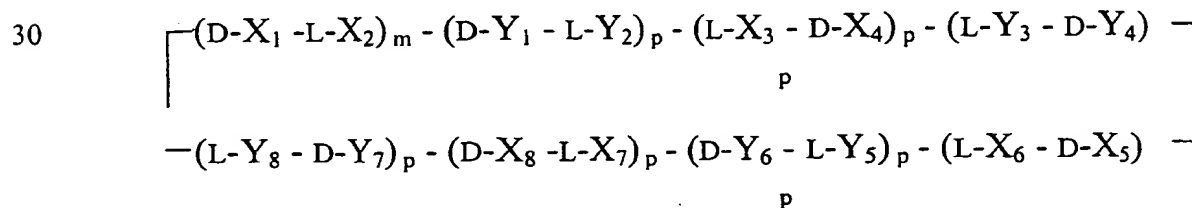10 are introduced, the variants are screened for biological activity.

In one embodiment, the cyclic peptides of the invention can have an amino acid sequence having formula I:

$$
\left[
\begin{array}{l}
(Y_1)_p\text{-}(X_1)_m\text{-}(Y_2)_p\text{-}(X_2)_p\text{-}(Y_3)_p\text{-}(X_3)_p\text{-}(Y_4)_p\text{-}(X_4)_p\text{-}(Y_5)_p\text{-}(X_5)_p \\
(X_{10})_p\text{-}(Y_{10})_p\text{-}(X_9)_p\text{-}(Y_9)_p\text{-}(X_8)_p\text{-}(Y_8)_p\text{-}(X_7)_p\text{-}(Y_7)_p\text{-}(X_6)_p\text{-}(Y_6)_p
\end{array}
\right]
$$

I

wherein:

   m is an integer ranging from 1 to 7;
20   each p is separately an integer ranging from 0 to 7;
   each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a
      polar D- or L-α-amino acid; and
   each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately
      nonpolar D- or L-α-amino acid; and
25   wherein the cyclic peptide has an even number of from four to about sixteen alternating D- and L-α amino acids.

In another embodiment, the cyclic peptides of the invention can have an amino acid sequence having formula II:

$$
\left[
\begin{array}{l}
(D\text{-}X_1\text{ -}L\text{-}X_2)_m - (D\text{-}Y_1 - L\text{-}Y_2)_p - (L\text{-}X_3 - D\text{-}X_4)_p - (L\text{-}Y_3 - D\text{-}Y_4)_p \\
(L\text{-}Y_8 - D\text{-}Y_7)_p - (D\text{-}X_8\text{ -}L\text{-}X_7)_p - (D\text{-}Y_6 - L\text{-}Y_5)_p - (L\text{-}X_6 - D\text{-}X_5)_p
\end{array}
\right]
$$

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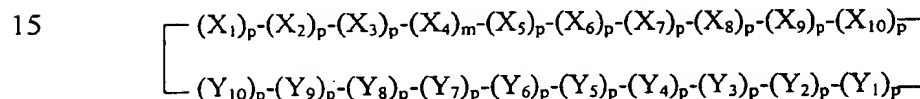II

wherein:

  m is an integer ranging from 1 to 7;

  each p is separately an integer ranging from 0 to 7;

  each X_1, X_2, X_3, X_4, X_5, X_6, X_7, and X_8 is separately a polar D- or

  L-α-amino acid;

  each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, and Y_8 is separately nonpolar D-

  or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

In yet another embodiment, the cyclic peptides of the invention can have

an amino acid sequence having formula III:

$$\left[\begin{array}{c} (X_1)_p\text{-}(X_2)_p\text{-}(X_3)_p\text{-}(X_4)_m\text{-}(X_5)_p\text{-}(X_6)_p\text{-}(X_7)_p\text{-}(X_8)_p\text{-}(X_9)_p\text{-}(X_{10})_p \\ (Y_{10})_p\text{-}(Y_9)_p\text{-}(Y_8)_p\text{-}(Y_7)_p\text{-}(Y_6)_p\text{-}(Y_5)_p\text{-}(Y_4)_p\text{-}(Y_3)_p\text{-}(Y_2)_p\text{-}(Y_1)_p \end{array} \right]$$

III

wherein:

  m is an integer ranging from 1 to 7;

  each p is separately an integer ranging from 0 to 7;

  each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a

      polar D- or L-α-amino acid;

  each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately

      nonpolar D- or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

In yet another embodiment, the cyclic peptide has an amino acid

sequence of formula IVa or IVb:

$$\text{D-}X_1 - (\text{L-}X_2 - \text{D-}X_3)_n - (\text{L-}Y_1 - \text{D-}Y_2)_m - \text{L-}Y_3 \qquad \text{IVa}$$

or

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$$\text{L-}X_1 - (\text{D-}X_2 - \text{L-}X_3)_n - (\text{D-}Y_1 - \text{L-}Y_2)_m - \text{D-}Y_3 \qquad \text{IVb}$$

wherein:

n is an integer ranging from 0 to 4;

m is an integer ranging from 1 to 7;

X_1, X_2 and X_3 are each a separate a polar amino acid;

Y_1, Y_2 and Y_3 are each a separate nonpolar amino acid; and

wherein the cyclic peptide has an even number of from four to about sixteen alternating D- and L-α amino acids.

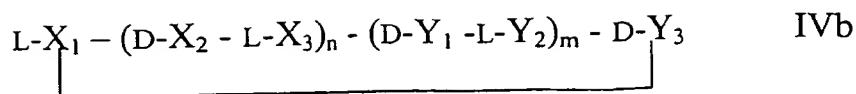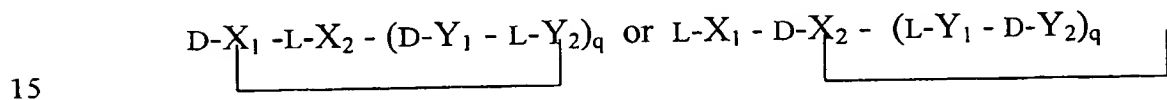In another embodiment, the cyclic peptide has an amino acid sequence of formula Va or Va:

$$\text{D-}X_1 - \text{L-}X_2 - (\text{D-}Y_1 - \text{L-}Y_2)_q \quad \text{or} \quad \text{L-}X_1 - \text{D-}X_2 - (\text{L-}Y_1 - \text{D-}Y_2)_q$$

Va   **Vb**

wherein:

q is an integer ranging from 2 to 7;

X_1 and X_2 are separately polar amino acids;

Y_1 and Y_2 are separately nonpolar amino acids.

The claimed cyclic peptides of the above formulae, however, exclude those composed entirely of nonpolar amino acids. Moreover, a peptide having any one of the following sequences may be excluded from one or more of the above formulae:

cyclo-[-(Gln- D-Ala)$_4$-]

cyclo-[-(Gln- D-Leu)$_4$-]

cyclo-[-(Gln- D-Val)$_4$-]

cyclo-[-(Phe- D-Leu)$_4$-]

cyclo-[-(Phe- D-Ala)$_4$-]

cyclo-[-(Gln- D-Ala-Glu- D-Ala)$_2$-]

cyclo-[-(Gln- D-Phe-Glu- D-Phe)$_2$-]

cyclo-[-(Gln- D-Ala-Glu- D-Ala)$_3$-]

cyclo-[-(Trp- D-Leu)$_3$-Gln- D-Leu-]

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cyclo-[-Gln- (D-Leu-Trp)4- D-Leu-]

cyclo-[-Lys-D-Leu-Lys-D-Leu-Lys-D-Leu-Lys-D-Leu];

cyclo-[-Arg- D-Leu -Arg- D-Leu- Arg- D-Leu-Arg- D-Leu -];

cyclo-[-Glu- D-Leu -Glu- D-Leu-Glu- D-Leu-Glu- D-Leu -].

5   Additionally, the following sequences may or may not be excluded from

the above formulae and associated peptides and compositions:

cyclo-[-D-Arg-Gln-D-Arg-Ala- D-Trp-Leu- D-Trp-Trp-];

cyclo-[-D-Arg-Gln-D-Arg-Leu-D-Trp-Leu- D-Trp-Trp-];

cyclo-[-D-Arg-Gln-D-Arg-Val-D-Trp-Leu- D-Trp-Trp-];

10   cyclo-[-D-Arg-Gln-D-Arg-Phe-D-Trp-Leu- D-Trp-Trp-]; or

cyclo-[-D-Arg-Gln-D-Arg-Trp-D-Trp-Leu- D-Trp-Trp-].

Cyclic peptides having any of the above sequences, however, including any of

the excluded sequences, may be included within or excluded from

15   pharmaceutical compositions and peptides of the present invention.

For example, the X amino acids in the above formulae can be D-serine,

D-threonine, D-asparagine, D-glutamine, D-aspartic acid, D-cysteine, D-glutamic

acid, D-histidine, D-homocysteine, D-arginine, D-lysine, D-hydroxylysine, D-

ornithine, L-serine, L-threonine, L-asparagine, L-glutamine, L-aspartic acid, L-

20   cysteine, L-glutamic acid, L-histidine, L-homocysteine, L-arginine, L-lysine, L-

hydroxylysine or L-ornithine, provided that the α-cyclic peptide has a sequence

of alternating D- and L-α-amino acids.

In some embodiments, one or more of the X amino acids are ionizable

amino acids. Such ionizable amino acids include, for example, D-aspartic acid,

25   D-glutamic acid, D-histidine, D-arginine, D-lysine, D-hydroxylysine, D-ornithine,

L-aspartic acid, L-glutamic acid, L-histidine, L-arginine, L-lysine, L-

hydroxylysine or L-ornithine.

The Y amino acids in the above formulae can be, for example, L-alanine,

L-valine, L-leucine, L-methionine, L-isoleucine, L-phenylalanine, L-tryptophan,

30   L-tyrosine, D-alanine, D-valine, D-leucine, D-methionine, D-isoleucine, D-

phenylalanine, D-tyrosine or D-tryptophan, provided that the α-cyclic peptide has

a sequence of alternating D- and L-α-amino acids. In other embodiments, the Y

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amino acids may be L-tryptophan, D-tryptophan, L-leucine or D-leucine, provided that the cyclic peptide has a sequence of alternating D- and L-amino acids.

The cyclic peptides of the present invention, for example, include any of SEQ ID NO:5, 7-22, 26-29, 40, 41, 43-55, 57, 58, 61-67, 72-77, 79-89, 91-93,

5   97-102, 107, 109-112, 114-117, 119-122, 125, 126, 128, 129, 133, 139, 140 or 141. In some embodiments the cyclic peptides employed are those with SEQ ID NO:8, 9, 12, 17, 18, 26, 29, 47-52, 61, 63, 67, 68, 72-77, 84, 85, 87-89, 91-93, 100, 102, 107, 111, 112, 119, 125 and 139. Formulations or compositions containing the present cyclic peptides can include a mixture of two or more

10   cyclic peptides.

The present isolated, purified peptides or variants thereof, can be synthesized *in vitro*, e.g., by the solid phase peptide synthetic method or by enzyme catalyzed peptide synthesis or with the aid of recombinant DNA technology. Solid phase peptide synthetic method is an established and widely

15   used method, which is described in references such as the following: Stewart et al., Solid Phase Peptide Synthesis, W. H. Freeman Co., San Francisco (1969); Merrifield, J. Am. Chem. Soc. 85 2149 (1963); Meienhofer in "Hormonal Proteins and Peptides," ed.; C.H. Li, Vol.2 (Academic Press, 1973), pp.48-267; and Bavaay and Merrifield, "The Peptides," eds. E. Gross and F. Meienhofer,

20   Vol.2 (Academic Press, 1980) pp.3-285. These peptides can be further purified by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an anion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; ligand

25   affinity chromatography; or crystallization or precipitation from non-polar solvent or nonpolar/polar solvent mixtures. Purification by crystallization or precipitation is preferred.

To identify highly active cyclic peptides that have little or no undesired toxicity for mammalian cells, individual cyclic peptides, or libraries of cyclic

30   peptides can be made and the individual cyclic peptides or cyclic peptides from those libraries can be screened for anti-microbial activity and toxicity. For example, libraries of peptides can be made using a one-bead-one-compound strategy provided by Lam et al. (97 Chem. Rev. 411-448 (1997) or synthesized

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on macrobeads by a split and pool method of Furka, et al. (37 Int. J. Pept. Prot. Res. 487-493(1991)). Mass spectrometric sequence analysis techniques enable rapid identification of every peptide within a given library. *See,* Biemann, K. 193 Methods Enzymol. 455 (1990). In general, synthetic operations, including

5   peptide cyclization, are performed on solid support to avoid laborious and difficult to automate solution-phase operations. Moreover, the final product of the synthesis regimen is generally sufficiently pure for biological assays without laborious purification procedures. Peptide yields from each synthesis can be sufficient for performing 50 to 100 assays. Rapid, automatable mass-

10   spectrometry-based peptide sequence analysis can be performed to identify peptide sequences that have high activity and to discard peptide sequences with low activity.

The synthetic approach employed can provide individually separable and identifiable peptide sequences to avoid the use of combinatorial library mixtures

15   and laborious deconvolution techniques. However, libraries of impure mixtures of peptides can also be generated for testing. Impure preparations of peptides can be used for quick screening of combinations of sequences. When a mixture of peptides shows activity, the peptides in the mixture can either be individually isolated and tested or pure peptides having sequences known to be present in the

20   impure mixture can be individually prepared and tested.

Salts of carboxyl groups of a peptide or peptide variant of the invention may be prepared in the usual manner by contacting the peptide with one or more equivalents of a desired base such as, for example, a metallic hydroxide base, e.g., sodium hydroxide; a metal carbonate or bicarbonate base such as, for

25   example, sodium carbonate or sodium bicarbonate; or an amine base such as, for example, triethylamine, triethanolamine, and the like.
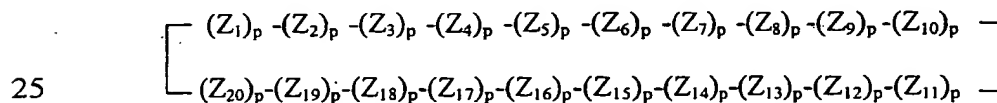
N-acyl derivatives of an amino group of the peptide or peptide variants may be prepared by utilizing an N-acyl protected amino acid for the final condensation, or by acylating a protected or unprotected peptide. O-acyl

30   derivatives may be prepared, for example, by acylation of a free hydroxy peptide or peptide resin. Either acylation may be carried out using standard acylating reagents such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N-acylation and O-acylation may be carried out together, if desired.

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Acid addition salts of the peptide or variant peptide, or of amino residues of the peptide or variant peptide, may be prepared by contacting the peptide or amine with one or more equivalents of the desired inorganic or organic acid, such as, for example, hydrochloric acid. Esters of carboxyl groups of the

5   peptides may also be prepared by any of the usual methods known in the art.

The invention also contemplates cyclic peptides composed of one or more β amino acids. Such β-amino acids can be substituted along their peptide backbones by one to two substitutents. Such substituents can include cylcoalkyl, cycloalkenyl, and heterocylic rings that encompass the α and β carbons of the β-

10   peptide backbone. These rings can be, for example, C_3 -C_8 cycloalkyl, cycloalkenyl or heterocyclic rings having one or more nitrogen atoms as the sole heteroatom, and can be substituted or unsubsituted. The substituents on the ring or on the α and β carbons of the β-peptide can be, for example, hydroxy, linear or branched C_1-C_6-alkyl, alkenyl, alkynyl; hydroxy-C_1-C_6-alkyl; amino-C_1-C_6-

15   · alkyl; C_1-C_6 -alkyloxy, C_1-C_6-alkoxy-alkyl; C_1-C_6 - amino; mono- or di-C_1-C_6- alkylamino; carboxamido; carboxamido-C_1-C_6-alkyl; sulfonamido; sulfonamido-C_1-C_6-alkyl, urea, cyano, fluoro, thio; C_1-C_6 -alkylthio; mono- or bicyclic aryl; mono- or bicyclic heteraryl having up to 5 heteroatoms selected from N, O, and S; mono- or bicyclic aryl- C_1-C_6 and heteroaryl -C_1-C_6 -alkyl and the like.

20   In one embodiment, the cyclic β-peptides of the invention can have an amino acid sequence of formula VI:

$$\left[\begin{array}{l} (Z_1)_p\,\text{-}(Z_2)_p\,\text{-}(Z_3)_p\,\text{-}(Z_4)_p\,\text{-}(Z_5)_p\,\text{-}(Z_6)_p\,\text{-}(Z_7)_p\,\text{-}(Z_8)_p\,\text{-}(Z_9)_p\,\text{-}(Z_{10})_p \\ (Z_{20})_p\text{-}(Z_{19})_p\text{-}(Z_{18})_p\text{-}(Z_{17})_p\text{-}(Z_{16})_p\text{-}(Z_{15})_p\text{-}(Z_{14})_p\text{-}(Z_{13})_p\text{-}(Z_{12})_p\text{-}(Z_{11})_p \end{array} \right]$$

25

VI

wherein:

each p is separately an integer ranging from 0 to 7;

30   each Z_1, Z_3, Z_5, Z_7, Z_9, Z_{11}, Z_{13}, Z_{15}, Z_{17}, and Z_{19} is separately a monosubstituted β-amino acid;

each Z_2, Z_4, Z_6, Z_8, Z_{10}, Z_{12}, Z_{14}, Z_{16}, Z_{18}, and Z_{20} is separately a disubstituted β-amino acid; and

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wherein the cyclic β-peptide has a sequence of from three to about ten homochiral β-amino acids.

Supramolecular Structures

5   According to the present invention the cyclic peptides provided herein are believed self-assemble into supramolecular structures. Self-assembly means that a collection of cyclic peptides can associate to form a supramolecular structure on or within a cellular membrane without the assistance of anything other than the components of the cellular membrane. In general, the physical

10   and chemical properties of the cellular membrane facilitate self-assembly of the cyclic peptides. The interaction between the components of microbial cellular membranes and the cyclic peptides determines whether the cyclic peptides are selective for those cellular membranes.

          Formation of supramolecular structures by the peptides of the invention

15   is supported by high-resolution imaging using cryo-electron microscopy, electron diffraction, Fourier-transform infrared spectroscopy, and molecular modeling. Supramolecular structures have been further characterized by IR spectroscopy, low-dose electron microscopy, and the analysis of electron diffraction patterns.

20   According to the present invention, cyclic peptide structures that are made up of an even number of alternating D- and L-amino acid residues are believed to adopt or sample a flat ring-shaped conformation in which all backbone amide functionalities lie approximately perpendicular to the plane of the ring structure. Similarly, cyclic peptides made up of β-amino acids can also

25   adopt a flat-ring structure. In this flat-ring conformation, it is believed that the peptide subunits can stack, under favorable conditions, to furnish a contiguous hydrogen bonded hollow tubular structure that is referred to herein as a nanotube (see Figures 1 and 7).

          For example, controlled acidification of alkaline solutions of peptide

30   SEQ ID NO:1 (cyclo[-(Gln-D-Ala-Glu D-Ala)$_3$-]) yielded rod shaped crystalline materials that appeared under transmission electron microscopy as organized bundles of tightly packed tubular structures. Low dose cryo microscopy,

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according to the method of M. Adrian et al. (308 *Nature* 32-36 (1984)) and of

R.A. Milligan et al. (13 *Ultramicroscopy* 1-10 (1984)) revealed longitudinal

striations with spacing of approximately 25 Å as expected for the center to center

spacing for closely packed tubular structures. Electron diffraction patterns

5   display axial spacing of 4.80 Å that is in agreement with the peptide stacking and

the formation of tight network of hydrogen bonded b-sheet type structure. The

meridonial spacing in the electron diffraction patterns display spacing of

12.67 ± 0.06 Å and 21.94 ± 0.05 Å characteristic of a hexagonal body centered

packing of nanotubes. Hexagonal lattice resulting from the close packing of

10   cylinders of radius r displays the characteristic two principle lattice planes of

radius r and r such as the one observed here (r=12.67 Å and r =21.94 Å). The

periodicity in this packing produces diffraction spots at $1/r$, $2/r$, and so on, and at

$1/r$, and $2/r$, and so on. The observed electron diffraction patterns on the

meridional axes extend to third order reflections (4.1 Å) signifying the ordered

15   and crystalline state of nanotube structures. The diffraction patterns also showed

a unit cell with an angle of 99° and no other symmetry than the center of

symmetry pursuant to Friedel's law.

A three-dimensional supramolecular structure model was built using the

parameters obtained from the electron diffraction patterns—unit cell with a=9.6

20   Å (2x4.80 Å for the antiparallel dimer), b=c=25.66 Å (2x12.67÷Cos9), α=120°,

and β=γ=99°. The model shows structure factors similar to the patterns

observed in the electron diffraction thus supporting the proposed three-

dimensional model. Involvement of intermolecular hydrogen bonding network

in a tube-like assembly is also supported by FT-IR spectroscopic analysis

25   according to the method of S. Krimm et al. (*Advances in Protein Chemistry*;

Anfinsen, C. B., Edsall, J. T.; Richards, F. M. Eds.; Academic Press: Orlando,

1986, pages 181-364). Nanotubes display characteristic IR features of a β-sheet

structure signified not only by the *amide I* bands at 1626 cm^{-1} and 1674 cm^{-1} and

an *amide II* band at 1526 cm^{-1}, but also by the observed NH stretching frequency

30   at 3291 cm^{-1} supporting formation of a tight network of hydrogen bonds.

The IR spectrum is similar to tubular structures that have been discovered

in nature. For example, nanotubular structures for some of the present peptides

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can be conceptually related to the structure of crystalline linear Gramicidin A that is known to form dimeric β-helical structures. Gramicidin A has *amide I* bands at 1630, 1685 cm^{-1}, an *amide II* band at 1539 cm^{-1}, and an NH stretching frequency at 3285 cm^{-1}. (V.M. Naik et al. in *Biophys. J.* (1986), vol. *49*, pages

5   1147-1154.) The observed frequency of NH stretching mode correlates to an average intersubunit distance of 4.76 Å that is in close agreement with the value of 4.80 Å obtained independently from the electron diffraction patterns.

The pore size, or internal diameter, of self-assembled nanotubes can be adjusted by the ring size of the peptide subunit employed. A twelve-residue

10   cyclic peptide structure, for example cyclo[-(Gln-D-Ala-Glu-D-Ala)$_3$-] (SEQ ID NO:1), has a diameter of about 13 Å. The eight residue cyclic peptide cyclo[-(Trp-D-Leu)$_3$-Gln-D-Leu-] (SEQ ID NO:2) has a diameter of approximately 7.5 Å in diameter. A cyclic peptide having SEQ ID NO:2 in synthetic phosphatidylcholine liposomes displays an FTIR *amide-I* band at 1624 cm^{-1} and

15   an observed N-H stretching frequency at 3272 cm^{-1} that support formation of a tight network of β-sheet-like hydrogen bonds with an average intersubunit distance of 4.7 Å to 4.8 Å.

The flat, ring-shaped cyclic peptides of the present invention are not only structurally predisposed toward intermolecular interaction, but are also

20   energetically favored to self-assemble on selected microbial cell membranes and permeabilize cells through formation of pores or other membrane destabilizing structures.

Formation of supramolecular structures that can permeabilize membranes was also inferred from proton transport activity. Vesicles were prepared having

25   pH 6.5 inside and pH 5.5 in the outside bulk solution. The collapse of the imposed pH gradient in these vesicles, upon formation of the putative transmembrane channel structure, was studied by monitoring the fluorescence intensity of an entrapped pH-sensitive dye. (V.E. Carmichael et al, in *J.Am. Chem. Soc.* (1989), vol. 111, pages 767-769). Addition of peptide cyclo[-(Trp-

30   D-Leu)$_3$-Gln-D-Leu-] (SEQ ID NO:2) to such vesicles suspensions causes a rapid collapse of the pH gradient.

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Unilamellar vesicles were prepared by the reverse-phase evaporation using DPPC, OPPC, cholesterol in the ratio of 1:1:2 in a solution containing 5(6)-carboxyfluorescein (20 mM in phosphate/saline buffer: 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 6.5) according to the method

5   of F. Szoka et al. in *Proc. Natl. Acad. Sci. USA* (1978), vol. 75, pages 4194-4198. Liposomes were then sized by multiple extrusions through Nucleopore® polycarbonate membranes (10 times, 50 psi, using 0.8 and 2x0.4 micron filter stacks) and the untrapped 5(6)-carboxyfluorescein was removed by size exclusion chromatography (Sephadex G-25 column 1x30 cm) using the same

10   phosphate/saline buffer according to the method of F. Olson et al. in *Biochim. Biophys. Acta* (1979), vol. 557, pages 9-23. Vesicles formed in this way are approximately 150 nanometer in diameter as determined by electron microscopy. (R.R.C. New, Ed. *Liposomes*, Oxford university Press, 1990). In each experiment, 70 ml of the stock vesicle solution (3.5×10^{-3} M in phospholipids)

15   was added to pH 5.5 buffer (1.3 ml, 137 mM NaCl, 2.6 mM KCl, 6.4 Na₂HPO₄, 1.4 KH₂PO₄) and placed in a 1 cm quartz cuvette inside a stirring thermojacketed sample holder of the fluorescence instrument and equilibrated at 25 °C for 15 minutes with gentle stirring. To the cuvette, through an injector port, 25 ml of the peptide dissolved in DMSO was added with continuous fluorescence

20   monitoring at 520 nm (excitation at 470 nm). The observed data were then normalized for comparison into the fractional change in fluorescence ($(I_0-I_t)/(I_0-I_\infty)$) (V.E. Carmichael et al, in *J.Am. Chem. Soc.* (1989), vol. 111, pages 767-769)).

Control studies, monitoring the release of carboxyfluorescein dye

25   entrapped in liposomes, indicated that the collapse of the pH gradient was not due to the rupturing of the liposomes nor due to the small amounts of organic solvents (<2% DMSO) employed in these studies. Furthermore, the control peptide cyclo[-(Gln-D-Leu)₄] that lacks the appropriate surface characteristics for partitioning into the liposomes, does not display any ion transport activity under

30   similar conditions. A second control peptide *cyclo*[-(Me N-D-Ala-Phe)₄-] that has the desirable hydrophobic surface characteristics but lacks the propensity for participating in extended hydrogen bonding network, was also designed and

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tested for ion transport activity. The ring structure of this peptide is N-methylated on one face. Such N-methylation does not adversely affect the ability of the peptide to interact with liposomal membranes but predisposes peptides toward a dimeric cylindrical structure that cannot span a normal

5   liposomal membrane. Thus, although the peptide has been shown to partition effectively into liposomes, it does not promote proton transport activity in the above vesicle experiments. Together, these experiments support the idea that not only are the side chains displayed on cyclic peptides important for membrane interaction, but also the peptide backbone should be able to participate in

10   extended intermolecular hydrogen bonding, for example, to facilitate membrane permeabilization.

Methods of Use

The present invention is directed to methods of treating or preventing

15   microbial infections in a mammal, as well as other animals, such as farm animals and birds. These methods include administering to the animal a therapeutically effective amount of a cyclic peptide of the present invention. Treatment of, or treating, microbial infections is intended to include the alleviation of or diminishment of at least one symptom typically associated with the infection.

20   The treatment also includes alleviation or diminishment of more than one symptom. Ideally, the treatment cures, e.g., substantially kills the microbes and/or eliminates the symptoms associated with the infection.

Microbial infections that can be treated by the present cyclic peptides include infections by any target microbial organisms that can infect a mammal or

25   other animal. Such target microbial organisms include essentially any single cell organism or parasite that has a cellular membrane and that can infect an animal, including mammals. For example, target microbial organisms include bacteria, fungi, yeast strains and other single cell organisms. Cyclic peptides are active against both gram-negative and gram-positive bacteria.

30   Hence, for example, infections of the following target microbial organisms can be treated by the present cyclic peptides: *Aeromonas* spp., *Bacillus* spp., *Bacteroides* spp., *Campylobacter* spp., *Clostridium* spp., *Enterobacter* spp., *Enterococcus* spp., *Escherichia* spp., *Gastrospirillum* sp.,

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Helicobacter spp., *Klebsiella* spp., *Salmonella* spp., *Shigella* spp.,

Staphylococcus spp., *Pseudomonas* spp., *Vibrio* spp., *Yersinia* spp., and the like.

Infections that can be treated by the present peptides include those associated

with staph infections (*Staphylococcus aureus*), typhus (*Salmonella typhi*), food

5   poisoning *(Escherichia coli*, such as O157:H7), bascillary dysentery (*Shigella

dysenteria*), pneumonia (*Psuedomonas aerugenosa* and/or *Pseudomonas

cepacia*), cholera (*Vivrio cholerae*), ulcers (*Helicobacter pylori*) and others. *E.

coli* serotype 0157:H7 has been implicated in the pathogenesis of diarrhea,

hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic

10   thrombocytopenic purpura (TTP). The peptides of the invention are also active

against drug-resistant and multiply-drug resistant strains of bacteria, for

example, multiply-resistant strains of *Staphylococcus aureus* and vancomycin-

resistant strains of *Enterococcus faecium* and *Enterococcus faecalis*.

Anti-microbial activity can be evaluated against these varieties of

15   microbes using methods available to one of skill in the art. Anti-microbial

activity, for example, is determined by identifying the minimum inhibitory

concentration (MIC) of a cyclic peptide of the present invention that prevents

growth of a particular microbial species. In one embodiment, anti-microbial

activity is the amount of the peptide that kills 50% of the microbes when

20   measured using standard dose or dose response methods.

The present invention also provides a method of evaluating

therapeutically effective dosages for treating a microbial infection with cyclic

peptides described and claimed herein that includes determining the minimum

inhibitory concentration of a cyclic peptide at which substantially no microbes

25   grow *in vitro*. Such a method permits calculation of the approximate amount of

cyclic peptide needed per volume to inhibit microbial growth or to kill 50% of

the microbes. Such amounts can be determined, for example, by standard

microdilution methods. For example, a series of microbial culture tubes

containing the same volume of medium and the substantially the same amount of

30   microbes are prepared, and an aliquot of cyclic peptide is added. The aliquot

contains differing amounts of cyclic peptide in the same volume of solution.

The microbes are cultured for a period of time corresponding to one to ten

generations and the number of microbes in the culture medium is determined.

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The optical density of the cultural medium can be used to estimate whether microbial growth has occurred – if no significant increase in optical density has occurred, then no significant microbial growth has occurred. However, if the optical density increases, then microbial growth has occurred. To determine

5 how many microbial cells remain alive after exposure to the cyclic peptide, a small aliquot of the culture medium can be removed at the time when the cyclic peptide is added (time zero) and then at regular intervals thereafter. The aliquot of culture medium is spread onto a microbial culture plate, the plate is incubated under conditions conducive to microbial growth and, when colonies appear, the

10 number of those colonies is counted.

According to the present invention, the cyclic peptides provided herein do not cause substantial or undesired toxicity against mammalian cells or the cells of otheranimals to be treated. Mammalian or bird red blood cell hemolysis is one way to measure whether a cyclic peptide can cause undesired toxicity

15 against mammalian cells or the cells of other animals to be treated. If a cyclic peptide can self-assemble by association with a mammalian or animal cell membrane, the membrane may be disrupted. Red blood cells are conveniently used to test for membrane disruption, because they undergo hemolysis, which can be detected as the release of hemoglobin from the cell. Hemolysis assays

20 can be performed by methods available to one of skill in the art. For example, after exposure to test compounds, the release of hemoglobin can be observed spectrophotometrically by observing the absorbance of light at wavelengths characteristic of hemoglobin, for example, at 543 nm. Control samples can be used, for example, the medium in which the cells are tested or maintained can

25 serve as a zero blank. A second control can be used to determine the absorbance value for 100% lysis or hemolysis that can be a sample that is identical to the test mammalian cell sample but which had been sonicated to completely disrupt the cells. Additionally hemolytic agents such as mellitin or a variety of detergents can also be used to establidh 100% hemolysis of test red blood cells.

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Screening Assays

Screening or other assays may be used to identify, confirm or evaluate cyclic peptides that can selectively interact with, rupture of kill a microbe or

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other cell type of interest. A wide variety of assays may be used for this purpose. In general, such an assay can involve contacting a microbe or other cell type of interest with at least one cyclic peptide and observing whether the cyclic peptide interacts with a microbe or other cell type of interest and/or has

5   deleterious effects upon that microbe or other cell type.

Methods available in the art can be used for determining whether the cyclic peptides of the invention, for example, interact with the membrane of a cell type of interest. For example, cyclic peptides can be labeled with a reporter molecule that permits detection of the peptide.   After labeling, the cyclic

10   peptides can be contacted with the cell type of interest for a time and under conditions that permit binding or association of the peptide to cellular membranes. The cells can be washed with physiological solutions to remove unbound or unassociated cyclic peptides, and the microbes or cells can then be observed to ascertain whether the reporter molecule is bound or associated with

15   the microbes, cells or cellular membranes. In another embodiment, one of skill in the art can test whether the cyclic peptide(s) can selectively penetrate the membranes of particular microbes or other selected cell types. This may be done, for example, by examining whether the reporter molecule remains associated with the cellular membranes of the microbe or cell type of interest or

20   whether the reporter molecule becomes associated with the interior of the microbe or cell type of interest.

Reporter molecules that can be employed include any detectable compound or molecule available to one of skill in the art that is conjugated directly or indirectly to a cyclic peptide of the invention. The label may itself be

25   detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition that is detectable or molecular epitopes for protein/antibody capture and detection.

Deleterious effects upon the microbe or cell type of interest can also be

30   detected as an indication of an interaction between a cyclic peptide of the invention and the microbe or cell type of interest. Such deleterious effects may be confirmed by any evidence that the cyclic peptide has had an adverse or cytotoxic effect upon the microbe or cell type of interest. For example, one of

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skill in the art can test whether the cyclic peptide(s) kill the cell type, or cause membrane depolarization or permeabilization of the membranes of the microbes or cell types of interest.

5   Of particular interest are screening assays for cyclic peptides that have low toxicity for normal human or other animal cells but that have good anti-microbial properties (depolarizing or permeabilizing microbial cell membranes, lysing or otherwise killing microbes). Such cyclic peptides may or may not interact with normal human or other animal cells so long as they have few toxic effects on such normal human or other animal cells.

10   Generally, in the testing of multiple peptides a plurality of assays are performed in parallel with different cyclic peptides, which may be introduced at different concentrations to obtain a differential response to the various concentrations. Typically, at least one control assay is included in the testing. Such a control can be a negative control involving exposure of the microbes or cells of interest to a physiologic solution containing no cyclic peptide. Another control can involve exposure of the microbe or cell type of interest to a cyclic peptide that has already been observed to adversely affect the microbe or cell type of interest, or a second cell type that is related to the microbe or cell type of interest. Another control can involve exposing a microbe or cell type of interest to a known therapeutic agent that has a desired affect on the microbe or cell type of interest, for example, an anti-microbial or cytotoxic agent with known efficacy at a particular concentration or dosage. One of skill in the art can readily select control compounds and conditions that facilitate such evaluations.

        Candidate cyclic peptides are obtained from a wide variety of sources including libraries of cyclic peptides generated as described herein. Cyclic peptides can also be individually or rationally designed and synthesized to have specific structural features selected by one of skill in the art.

        Any cell type available to one of skill in the art can be screened by these methods. For example, any microbial or mammalian or animal cell type can be screened to assess whether the cyclic peptides of the invention can selectively or non-selectively interact therewith. Such microbial cell types include any single cell organism that is capable of autonomous replication. Examples include any bacterial, fungal and yeast cell types. Mammalian or other animal cell types can

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also be screened to ascertain whether the peptides of the invention interact therewith and/or to determine or confirm whether the peptides of the invention do not interact, bind, lyse, kill or otherwise adversely affect the viability of the mammalian or other animal cell type of interest.

5   In one embodiment, mammalian red blood cells are screened with the cyclic peptides to ascertain whether the cyclic peptides have an adverse effect on the red blood cells. The membranes of red blood cells tend to be more sensitive to lysis than many other mammalian cell types. Hence, red blood cells are a useful cell type for quickly screening whether a cyclic peptide would be

10   expected to have any adverse effects on these or other mammalian cell types. Methods of screening for mammalian cell lysis are available in the art. For example, using procedures described herein, red blood cells can be tested to ascertain whether hemolysis has occurred upon exposure to at least one cyclic peptide of the invention. When it is established that a cyclic peptide causes little

15   or no undesired hemolysis of red blood cells, it may be tested against other mammalian cell types or used for *in vivo* testing in standard animal models.

Conditions for screening cyclic peptides include conditions that are used by one of skill in the art to grow, maintain or otherwise culture cell types of interest. Cell types of interest should be assayed under conditions where they

20   would be healthy but for the presence of the cyclic peptide(s). Controls can be performed where the cell types are maintained under the selected culture conditions and not exposed to a cyclic peptide, to assess whether the culture conditions influenced the viability of the cells. One of skill in the art can also perform the assay on cells that have been washed in simple physiological

25   solutions, such as buffered saline, to eliminate, or test for, any interaction between the cyclic peptides or cells and the components in the culture media. However, culture conditions for the assays generally include providing the cells with the appropriate concentration of nutrients, physiological salts, buffers and other components typically used to culture or maintain cells of the selected type.

30   A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, albumin, serum (e.g. fetal calf serum) that are used to mimic the physiologic state of the cell types of interest.

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Conditions and media for culturing, growing and maintaining mammalian cells and bacterial or other microbial cells are available to one of skill in the art.

The selected reagents and components are added to the assay in the order selected by one of skill in the art. In general, the cyclic peptides are added last to start the assay. Assays are performed at any suitable temperature, typically between 4 °C and 40° C. Temperatures generally range from about room temperature (about 20 °C) to about 37°C. Incubation periods are selected to ascertain the optimal range of activity or to insure that the cyclic peptides do not adversely affect unwanted cell types. However, incubation times can be optimized to facilitate rapid high-throughput screening. Typically incubation times are between about 1 minute and about 24 hours, other times range from about 5 minutes to about 8 hours.

Cyclic peptides having the desired selectivity and activity during *in vitro* screening or evaluation may be tested for activity and/or lack of toxicity *in vivo* in an appropriate animal model. Such animal models include mice, rats, rabbits, cats, dogs, pigs, goats, cattle or horses. For example, the mouse and the rat are convenient animal models for testing whether cyclic peptides of the invention have toxic effects and/or to determine whether the cyclic peptides can combat a microbial infection.

One of skill in the art can readily perform *in vivo* screening of the cyclic peptides of the invention. For toxicity testing, a series of cyclic peptides at different test dosages can be separately administered to different animals. A single dose or a series of dosages can be administered to the animal. A test period is selected that permits assessment of the effects of the peptide(s) on the animal. Such a test period may run from about one day to about several weeks or months.

The effect of a cyclic peptide(s) on an animal can be determined by observing whether the peptide adversely affects the behavior (e.g., lethargy, hyperactivity) and physiological state of the animal over the course of test period. The physiological state of the animal can be assessed by standard procedures. For example, during the test period one of skill in the art can draw blood and collect other bodily fluids to test, for example, for various enzymes,

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proteins, metabolites, and the like. One of skill in the art can also observe whether the animal has bloating, loss of appetite, diarrhea, vomiting, blood in the urine, loss of consciousness, and a variety of other physiological problems. After the test period, the animal can be sacrificed and anatomical, pathological,

5   histological and other studies can be performed on the tissues or organs of the animal.

In general, to determine whether one or more cyclic peptides of the invention can combat a microbial infection, mice or other test animals are infected with the selected microbe and a selected test dosage of one or more

10 cyclic peptides is administered thereafter at predetermined elapsed time periods or intervals. Test animals are observed over the course of several days to weeks to ascertain whether the cyclic peptide protects the animals from the microbial infection. At the end of the test period, the test animals can be sacrificed and examined to ascertain whether the cyclic peptide has optimally protected the test

15 animals from infection and/or to determine whether any adverse side effects have occurred.

Prior to administration, the microbe can be washed in simple physiological solutions to remove toxins or other components that may adversely affect the mice. For example, microbial cells can be grown at 37 °C with

20 agitation for 12 hours to a stationary phase. Microbial cells can be collected by centrifugation, washed twice with saline or phosphate buffered saline (PBS) and resuspended or diluted to a convenient cell density, for example, a cell density of about of about $3\text{-}5\text{x}10^7$ cfu/ml. Several animals to be tested with varying amounts of each peptide five are infected with a small volume of the microbe

25 (e.g. about 0.1 to about 1 ml). Infection can be performed orally, intraperitoneally, intravenously or by some other route selected by one of skill in the art. After infection, the animals are allowed to rest for a short time and then each animal is treated with a different dose of peptide. The animals are monitored for several days to weeks.

30   Controls are used to establish the effects of the microbe when the cyclic peptide is not administered. Other controls can also be performed, for example, the safety and efficacy of the present cyclic peptides can be compared to that of

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known anti-microbial agents (e.g., penicillin, kanamycin, vancomycin, erythromycin, etc.).

5   The invention further provides a method of identifying or evaluating a D-, L-α-cyclic peptide or a β-peptide capable of selective association with a target biomolecule. Such target biomolecules can include, for example, intracellular, extracellular or membrane-associated proteins, enzymes, nucleic acids, receptors, organelles and the like. This method can involve contacting a solution of cyclic peptides with the target biomolecule under hydrogen bond-promoting conditions and determining whether the peptides selectively associate with the

10   desired biomolecules and possess biological activity of interest.

The target biomolecule can be displayed, for example, on the surface of a living cell, on the surface of a genetically engineered cell or on the surface of a liposome. Alternatively, the peptide can be contacted with the target biomolecule under other desired assay conditions available to one of skill in the

15   art.

Cyclic peptides having good anti-microbial properties *in vitro* and/or *in vivo* that also have substantially no undesired toxicity against unwanted cell types are particularly good candidates for the prepartion of appropriate dosage forms, as described in more detail below.

20

Dosages, Formulations and Routes of Administration for the Peptides

The peptides of the invention, including their salts, are administered so as to achieve a reduction in at least one symptom associated with an infection, indication or disease, or a decrease in the amount of antibody associated with the

25   indication or disease.

To achieve the desired effect(s), the peptide, a variant thereof or a combination thereof, may be administered as single or divided dosages, for example, of at least about 0.01 mg/kg to about 500 to 750 mg/kg, of at least about 0.01 mg/kg to about 300 to 500 mg/kg, at least about 0.1 mg/kg to about

30   100 to 300 mg/kg or at least about 1 mg/kg to about 50 to 100 mg/kg of body weight, although other dosages may provide beneficial results. The amount administered will vary depending on various factors including, but not limited

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to, the cyclic peptide chosen, the disease, the weight, the physical condition, the health, the age of the mammal, whether prevention or treatment is to be achieved, and if the peptide is chemically modified. Such factors can be readily determined by the clinician employing animal models or other test systems that

5   are available in the art.

Administration of the therapeutic agents in accordance with the present invention may be in a single dose, in multiple doses, in a continuous or intermittent manner, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or

10   prophylactic, and other factors known to skilled practitioners. The administration of the peptides of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated.

To prepare the composition, peptides are synthesized or otherwise

15   obtained, purified as necessary or desired and then lyophilized and stabilized. The peptide can then be adjusted to the appropriate concentration, and optionally combined with other agents. The absolute weight of a given peptide included in a unit dose can vary widely. For example, about 0.01 to about 2 g, or about 0.1 to about 500 mg, of at least one peptide of the invention, or a plurality of

20   peptides specific for a particular cell type can be administered. Alternatively, the unit dosage can vary from about 0.01 g to about 50 g, from about 0.01 g to about 35 g, from about 0.1 g to about 25 g, from about 0.5 g to about 12 g, from about 0.5 g to about 8 g, from about 0.5 g to about 4 g, or from about 0.5 g to about 2 g.

25   Daily doses of the cyclic peptides of the invention can vary as well. Such daily doses can range, for example, from about 0.1 g/day to about 50 g/day, from about 0.1 g/day to about 25 g/day, from about 0.1 g/day to about 12 g/day, from about 0.5 g/day to about 8 g/day, from about 0.5 g/day to about 4 g/day, and from about 0.5 g/day to about 2 g/day.

30   Thus, one or more suitable unit dosage forms comprising the therapeutic peptides of the invention can be administered by a variety of routes including oral, parenteral (including subcutaneous, intravenous, intramuscular and intraperitoneal), rectal, dermal, transdermal, intrathoracic, intrapulmonary and

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intranasal (respiratory) routes. The therapeutic peptides may also be formulated

for sustained release (for example, using microencapsulation, see WO 94/ 07529,

and U.S. Patent No.4,962,091). The formulations may, where appropriate, be

conveniently presented in discrete unit dosage forms and may be prepared by

5   any of the methods well known to the pharmaceutical arts. Such methods may

include the step of mixing the therapeutic agent with liquid carriers, solid

matrices, semi-solid carriers, finely divided solid carriers or combinations

thereof, and then, if necessary, introducing or shaping the product into the

desired delivery system.

10   When the therapeutic peptides of the invention are prepared for oral

administration, they are generally combined with a pharmaceutically acceptable

carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage

form. For oral administration, the peptides may be present as a powder, a

granular formulation, a solution, a suspension, an emulsion or in a natural or

15   synthetic polymer or resin for ingestion of the active ingredients from a chewing

gum. The active peptides may also be presented as a bolus, electuary or paste.

Orally administered therapeutic peptides of the invention can also be formulated

for sustained release, e.g., the peptides can be coated, micro-encapsulated, or

otherwise placed within a sustained delivery device. The total active ingredients

20   in such formulations comprise from 0.1 to 99.9% by weight of the formulation.

        By "pharmaceutically acceptable" it is meant a carrier, diluent, excipient,

and/or salt that is compatible with the other ingredients of the formulation, and

not deleterious to the recipient thereof.

        Pharmaceutical formulations containing the therapeutic peptides of the

25   invention can be prepared by procedures known in the art using well-known and

readily available ingredients. For example, the peptide can be formulated with

common excipients, diluents, or carriers, and formed into tablets, capsules,

solutions, suspensions, powders, aerosols and the like. Examples of excipients,

diluents, and carriers that are suitable for such formulations include buffers, as

30   well as fillers and extenders such as starch, cellulose, sugars, mannitol, and

silicic derivatives. Binding agents can also be included such as carboxymethyl

cellulose, hydroxymethylcellulose, hydroxypropyl methylcellulose and other

cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone. Moisturizing

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agents can be included such as glycerol, disintegrating agents such as calcium carbonate and sodium bicarbonate. Agents for retarding dissolution can also be included such as paraffin. Resorption accelerators such as quaternary ammonium compounds can also be included. Surface active agents such as cetyl

5   alcohol and glycerol monostearate can be included. Adsorptive carriers such as kaolin and bentonite can be added. Lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols can also be included. Preservatives may also be added. The compositions of the invention can also contain thickening agents such as cellulose and/or cellulose derivatives. They

10   may also contain gums such as xanthan, guar or carbo gum or gum arabic, or alternatively polyethylene glycols, bentones and montmorillonites, and the like.

     For example, tablets or caplets containing the cyclic peptides of the invention can include buffering agents such as calcium carbonate, magnesium oxide and magnesium carbonate. Caplets and tablets can also include inactive

15   ingredients such as cellulose, pregelatinized starch, silicon dioxide, hydroxy propyl methyl cellulose, magnesium stearate, microcrystalline cellulose, starch, talc, titanium dioxide, benzoic acid, citric acid, corn starch, mineral oil, polypropylene glycol, sodium phosphate, zinc stearate, and the like. Hard or soft gelatin capsules containing at least one cyclic peptide of the invention can

20   contain inactive ingredients such as gelatin, microcrystalline cellulose, sodium lauryl sulfate, starch, talc, and titanium dioxide, and the like, as well as liquid vehicles such as polyethylene glycols (PEGs) and vegetable oil. Moreover, enteric-coated caplets or tablets containing one or more peptides of the invention are designed to resist disintegration in the stomach and dissolve in the more

25   neutral to alkaline environment of the duodenum.

     The therapeutic peptides of the invention can also be formulated as elixirs or solutions for convenient oral administration or as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous, intraperitoneal or intravenous routes. The pharmaceutical formulations of the

30   therapeutic peptides of the invention can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension or salve.

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Thus, the therapeutic peptides may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers. As noted above,

5 preservatives can be added to help maintain the shelve life of the dosage form. The active peptides and other ingredients may form suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active peptides and other ingredients may be in powder form, obtained by aseptic

10 isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable carriers, vehicles and adjuvants that are well known in the art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable

15 from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol," polyglycols and polyethylene glycols, C_1-C_4 alkyl esters of short-chain acids, ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol," isopropyl

20 myristate, animal, mineral and vegetable oils and polysiloxanes.

It is possible to add, if necessary, an adjuvant chosen from antioxidants, surfactants, other preservatives, film-forming, keratolytic or comedolytic agents, perfumes, flavorings and colorings. Antioxidants such as t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol and its

25 derivatives can be added.

Also contemplated are combination products that include one or more cyclic peptides of the present invention and one or more other anti-microbial agents. For example, a variety of antibiotics can be included in the pharmaceutical compositions of the invention, such as aminoglycosides (e.g.,

30 streptomycin, gentamicin, sisomicin, tobramycin and amicacin), ansamycins (e.g. rifamycin), antimycotics (e.g. polyenes and benzofuran derivatives), β-lactams (e.g. penicillins and cephalosporins), chloramphenical (including

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thiamphenol and azidamphenicol), linosamides (lincomycin, clindamycin), macrolides (erythromycin, oleandomycin, spiramycin), polymyxins, bacitracins, tyrothycin, capreomycin, vancomycin, tetracyclines (including oxytetracycline, minocycline, doxycycline), phosphomycin and fusidic acid.

5   Additionally, the peptides are well suited to formulation as sustained release dosage forms and the like. The formulations can be so constituted that they release the active peptide, for example, in a particular part of the intestinal or respiratory tract, possibly over a period of time. Coatings, envelopes, and protective matrices may be made, for example, from polymeric substances, such

10   as polylactide-glycolates, liposomes, microemulsions, microparticles, nanoparticles, or waxes. These coatings, envelopes, and protective matrices are useful to coat indwelling devices, e.g., stents, catheters, peritoneal dialysis tubing, draining devices and the like.

           For topical administration, the therapeutic agents may be formulated as is

15   known in the art for direct application to a target area. Forms chiefly conditioned for topical application take the form, for example, of creams, milks, gels, dispersion or microemulsions, lotions thickened to a greater or lesser extent, impregnated pads, ointments or sticks, aerosol formulations (e.g., sprays or foams), soaps, detergents, lotions or cakes of soap. Other conventional forms

20   for this purpose include wound dressings, coated bandages or other polymer coverings, ointments, creams, lotions, pastes, jellies, sprays, and aerosols. Thus, the therapeutic peptides of the invention can be delivered via patches or bandages for dermal administration. Alternatively, the peptide can be formulated to be part of an adhesive polymer, such as polyacrylate or acrylate/vinyl acetate

25   copolymer. For long-term applications it might be desirable to use microporous and/or breathable backing laminates, so hydration or maceration of the skin can be minimized. The backing layer can be any appropriate thickness that will provide the desired protective and support functions. A suitable thickness will generally be from about 10 to about 200 microns.

30   Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilizing agents, dispersing agents,

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suspending agents, thickening agents, or coloring agents. The active peptides can also be delivered via iontophoresis, e.g., as disclosed in U.S. Patent Nos. 4,140,122; 4,383,529; or 4,051,842. The percent by weight of a therapeutic agent of the invention present in a topical formulation will depend on various

5   factors, but generally will be from 0.01% to 95% of the total weight of the formulation, and typically 0.1-85% by weight.

Drops, such as eye drops or nose drops, may be formulated with one or more of the therapeutic peptides in an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents or suspending

10   agents. Liquid sprays are conveniently delivered from pressurized packs. Drops can be delivered via a simple eye dropper-capped bottle, or via a plastic bottle adapted to deliver liquid contents dropwise, via a specially shaped closure.

The therapeutic peptide may further be formulated for topical administration in the mouth or throat. For example, the active ingredients may

15   be formulated as a lozenge further comprising a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the composition in an inert base such as gelatin and glycerin or sucrose and acacia; and mouthwashes comprising the composition of the present invention in a suitable liquid carrier.

The pharmaceutical formulations of the present invention may include, as

20   optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are available in the art. Examples of such substances include normal saline solutions such as physiologically buffered saline solutions and water. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical

25   formulations of the present invention include water and physiologically acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0.

The peptides of the invention can also be administered to the respiratory tract. Thus, the present invention also provides aerosol pharmaceutical

30   formulations and dosage forms for use in the methods of the invention. In general, such dosage forms comprise an amount of at least one of the agents of the invention effective to treat or prevent the clinical symptoms of a specific infection, indication or disease. Any statistically significant attenuation of one

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or more symptoms of an infection, indication or disease that has been treated pursuant to the method of the present invention is considered to be a treatment of such infection, indication or disease within the scope of the invention.

Alternatively, for administration by inhalation or insufflation, the

5   composition may take the form of a dry powder, for example, a powder mix of the therapeutic agent and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, e.g., gelatine or blister packs from which the powder may be administered with the aid of an inhalator, insufflator, or a metered-dose

10   inhaler (see, for example, the pressurized metered dose inhaler (MDI) and the dry powder inhaler disclosed in Newinan, S. P. in Aerosols and the Lung, Clarke, S. W. and Davia, D. eds., pp. 197-224, Butterworths, London, England, 1984).

Therapeutic peptides of the present invention can also be administered in

15   an aqueous solution when administered in an aerosol or inhaled form. Thus, other aerosol pharmaceutical formulations may comprise, for example, a physiologically acceptable buffered saline solution containing between about 0.1 mg/ml and about 100 mg/ml of one or more of the peptides of the present invention specific for the indication or disease to be treated. Dry aerosol in the

20   form of finely divided solid peptide or nucleic acid particles that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. Peptides of the present invention may be formulated as dusting powders and comprise finely divided particles having an average particle size of between about 1 and 5 μm, alternatively between 2 and 3 μm. Finely divided

25   particles may be prepared by pulverization and screen filtration using techniques well known in the art. The particles may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a powder. It will be appreciated that the unit content of active ingredient or ingredients contained in an individual aerosol dose of each dosage form need not

30   in itself constitute an effective amount for treating the particular infection, indication or disease since the necessary effective amount can be reached by administration of a plurality of dosage units. Moreover, the effective amount

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may be achieved using less than the dose in the dosage form, either individually, or in a series of administrations.

For administration to the upper (nasal) or lower respiratory tract by inhalation, the therapeutic peptides of the invention are conveniently delivered

5   from a nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered

10   amount. Nebulizers include, but are not limited to, those described in U.S. Patent Nos. 4,624,251; 3,703,173; 3,561,444; and 4,635,627. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co., (Valencia, CA). For intra-nasal

15   administration, the therapeutic agent may also be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker).

Furthermore, the active ingredients may also be used in combination with other therapeutic agents, for example, pain relievers, anti-inflammatory agents,

20   antihistamines, bronchodilators and the like, whether for the conditions described or some other condition.

The present invention further pertains to a packaged pharmaceutical composition for controlling microbial infections such as a kit or other container. The kit or container holds a therapeutically effective amount of a pharmaceutical

25   composition for controlling microbial infections and instructions for using the pharmaceutical composition for control of the microbial infection. The pharmaceutical composition includes at least one cyclic peptide of the present invention, in a therapeutically effective amount such that microbial infection is controlled.

30   The invention is further illustrated by the following non-limiting Examples.

Example 1.   Materials and Methods.

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Solid Phase Peptide Synthesis

Solvents and reagents: Acetonitrile (ACN, optima grade), dichloromethane (DCM, ACS grade), N,N-dimethylformamide (DMF, sequencing grade), diethylether (Et$_2$O, ACS grade), N,N-diisopropylethylamine

5   (DIEA, peptide synthesis grade) were purchased from Fisher and used without further purification. Trifluoroacetic acid (TFA, New Jersey Halocarbon), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, Richelieu Biotechnologies), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP, Novabiochem) were used as

10   obtained. Commercially available amino acids and resins were used as obtained from Bachem, Novabiochem or Advanced Chemtech. The side-chain protections were as follows. For Fmoc synthesis: Arg (Pbf), His (Boc), Lys (Boc), Ser (t-Bu) and Thr (t-Bu). All other chemicals were used as obtained from Aldrich, Acros, Sigma or Fluka.

15   *Peptide synthesis and cyclization:* Linear protected peptides were synthesized on polystyrene solid support using a trytil-functionalized resin and cyclized in the solution phase. The first Fmoc-protected amino acid was loaded onto a Cl-trytil-resin via its α-carboxylate group following standard procedures. Briefly, dry Fmoc-amino acid (1.2 eq. with respect to resin loading) was

20   dissolved in DCM (dried over NaHCO$_3$, 20 mL/g of resin) and DIEA (4 eq.). This mixture was added to a fresh commercially available Cl-trytil resin (loading 0.6-1.2 mmol/g) and continuously shaken for 2 hour, followed by sequential washings with DCM (3x20 mL), MeOH/DIEA/DCM (1:2:17, 3x15 mL) and DCM (3x20 mL). Loading of the first amino acid was estimated by removing

25   the Fmoc group with base from a measured amount of resin and then measuring the UV absorption and concentraion of the product in a known sample volume. The rest of the amino acids were sequentially introduced following standard Fmoc protocols. J. M. a. Y. Stewart, J. D., Solid Phase Peptide Synthesis 1984. After the last coupling, N-terminal Fmoc group was removed by treatment with

30   20% Piperidine/DMF. The peptide containing resin was washed with DMF (3x20 mL), then with DCM (3x20 mL). The side chain protected linear peptide was cleaved from the resin by sequential shaking with several portions (10-20)

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of TFA/DCM mixture (1/99) collecting the solution into a flask, containing

pyridine (2 mL) and MeOH (5 mL). The completion of the cleavage is achieved

when the resin changed its color to dark red. Then the resin was washed with

DCM (2x10 mL) and MeOH (2x10 mL). These washings were combined with

5   collected TFA cleavage solutions. The solvent was evaporated under vacuum (1

mm Hg) and the purity of the linear peptide was assessed by MALDI-MS and

HPLC. In most cases, purity of the crude linear peptide allowed cyclization

without prior purification. Otherwise, linear peptides were purified by reverse

phase high performance liquid chromatography (RP-HPLC/C4 or C18, 0.1%

10   TFA in ACN/H$_2$O).

Cyclization was performed in DMF at a peptide concentration of 1-5 mM

using a mixture of PyBOP (5 eq. with respect to crude peptide) and DIEA (40

eq.). The amount of DIEA was adjusted to achieve an apparent pH 9-10, which

was assessed by applying a drop of reaction mixture to a wet pH paper.

15   Reaction was followed by MALDI-MS and HPLC and in most cases it was

complete in less than 2 h. Then DMF was removed by evaporation under

vacuum (1 mm Hg) at temperatures less that 30 °C and the residue was dried

under vacuum (0.1 mm Hg) overnight.

For deprotection of the cyclic peptide side chains, the dried crude peptide

20   was dissolved in a mixture of TFA/PhOH/H$_2$O/thioanisole/EDT/TIS

(81.5:5:5:2.5:1) (~ 100 mL/g of peptide) at rt for 1-3 hours. The completion of

the reaction was followed by HPLC and MALDI-MS. The TFA solution was

concentrated by 5 times by evaporation under vacuum (1 mm Hg), from which

the peptide was precipitated by adding it to an ice-cold Et$_2$O. The purity of dried

25   crude peptides was assessed by HPLC and MALDI-MS. The crude peptides can

be partially purified by dissolving in boiling ACN/water/HCl mixture

(30/70/0.1) and cooling the turbid solution in a fridge. In case of peptide high

solubility in this mixture, the precipitate can be obtained by adding acetone (3

vol. eq.) to the above solution. Further purification was achieved by preparative

30   reverse phase HPLC (C4, radial compression column, Waters) using, for

example, a gradient of eluent A (0.1 % HCl in 99% H$_2$O/1% ACN (v/v)) and

eluent B (0.07 % HCl in 90% ACN/10% H$_2$O (v/v)) using a flow rate of 24

ml/min.

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Combinatorial Peptide Synthesis: Mixture Libraries

Materials for peptide synthesis by Boc chemistry were purchased from a variety of commercial sources. For the synthesis of the first generation libraries

5 of peptides, N-Boc-α-Fmoc-glutamic acid was loaded onto methyl-benzhydrylamine (MBHA) resin through its side chain carboxylate, then the resin was split into four equimolar fractions of 0.25 mmol each for the rest of the synthesis. To each fraction was coupled one of four N-Boc-protected *D*-amino acids, lysine, arginine, glutamic acid, or serine (4 equivalents), using (*O*-(7-

10 azabenzotriazol-1-yl)-1,1,3,3-) tetramethyluronimum hexafluorophosphate (HATU, 4 equivalents), 1-hydroxy-7-azabenzotriazole (HOAT, 4 equivalents), and diisopropylethylamine (DIEA, 6 equivalents) in DMF. To positions 3 through 7 were coupled a mixture of N-Boc-protected alanine, leucine, valine, phenylalanine, and tryptophan, at a molar ratio of 1.35: 1.97: 4.47: 1: 1 (10

15 equivalents versus resin loading) to compensate for the difference in the coupling efficiencies (Pinilla, C.; Appel, J. R.; Blondelle, S. E.; Dooley, C. T.; Eichler, J.; Ostresh, J. M.; Houghten, R. A. "Versatility of positional scanning synthetic combinatorial libraries for the identification of individual compounds." *Drug Dev. Res.* 1994, *33*, 133-145).

20 Standard procedures were utilized for peptide synthesis except that the odd positions contained amino acids of *L*-chirality and the even positions amino acids with *D*-chirality. In position 8, a mixture of N-Boc-protected *D*-amino acids, lysine, arginine, glutamic acid, and serine, at a molar ratio of 2.24: 2.34: 1.31: 1 was coupled (10 equivalents versus resin loading). After removal of the

25 N-terminal Boc from the peptide chain with neat TFA, the α-Fmoc on the carboxyl terminus of the glutamic acid was removed with 30% piperidine in DMF to allow cyclization of the peptide on the resin, using HATU (2 equivalents), HOAT (2 equivalents), and DIEA (4 equivalents) in DMF/DMSO and/or benzotriazole-1-yl-oxy-trix-pyrrolidino-phosphonium

30 hexafluorophosphate (PyBOP, 1 equivalent), N-hydroxybenzotriazole, (HOBT, 1 equivalent), and DIEA (2 equivalents) in dry 2M LiBr/tetrahydrofuran over a period of 24 - 48 hours. Peptides were cleaved by standard high HF cleavage

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procedure, washed with ether then extracted with 10% acetic acid followed by DMF. The extracts were pooled and lyophilized.

The specified amino acids of the peptide library sequences showing the greatest biological activity (lowest MIC value) were retained for the generation

5   of the next set of libraries. Subsequent generations of peptide libraries were synthesized in a similar fashion, with the splitting of the resin after the coupling of the specified amino acids determined from the previous generation. The peptides of the combinatorial libraries were identified by electrospray-mass spectrometry (ES-MS) or MALDI-TOF mass spectrometry.

10   When a peptide library, peptide pool or crude preparation of a peptide showed activity, individual peptides were re-synthesized, HPLC-purified and tested again for activity. For example, a crude preparation of a peptide having a low minimum inhibitory concentration value, *cyclo*[*D*-Arg-*L*-Gln-*D*-Arg-*L*-Trp-*D*-Trp-*L*-Leu-Trp-*L*-Trp] (SEQ ID NO:10), was resynthesized, HPLC-purified,

15   and tested for anti-microbial activity, and was found to have biological activity similar to the crude.

Single-Compound per Bead Combinatorial Cyclic Peptide Libraries

Cyclic *D, L*-α-peptide combinatorial libraries were prepared using a one-

20   bead-one-compound strategy on macrobeads by a split and pool method. *See,* K.S. Lam, M. Lebl, V. Krchnak, "The 'one-bead-one-compound' combinatorial library method," *Chem. Rev.* 1997, 97, 411-448. Each bead contained a single sequence and was dispersed into microtitre plates using a density of one bead per well. Cleavage of the peptide from a single bead provided about 70-80 μg of

25   peptide per well. This amount of peptide could be used for approximately 100 *in vitro* anti-microbial assays. Mass spectrometric peptide sequencing strategies were used for rapid identification of selected peptide species within a given library.

Solid-phase peptide synthesis was performed on polystyrene macrobeads

30   functionalized with a TFA-labile trityl linker, which considerably facilitated the synthesis, handling, solid-phase cyclization, and final side chain deprotection and peptide isolation. The growing peptide chain was linked through the first

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amino acid side chain (for example lysine or histidine) to the trityl moiety

allowing for selective "head-to-tail" cyclization of the completed peptide

sequence on solid support. The α-carboxyl group of the first N-α-Fmoc amino

acid was protected as an allyl ester. Resin loading and peptide chain elongation

5   was performed under standard Fmoc solid phase peptide synthesis conditions

using chlorotrityl polystyrene macrobead resin (500-560 um, Peptides

International) as the solid support, with HBTU as a coupling reagent and 20%

piperidine in DMF for Fmoc deprotection. After completion of the final amino

acid coupling, the resin was exposed to palladium tetrakis(triphenylphosphine)

10   and N-methyl morpholine to remove the C-terminal allyl protecting group.

Subsequent N-terminal Fmoc deprotection followed directly by cyclization with

PyBop®, provided the desired cyclic peptide generally in high yields. Single

macrobeads was dispensed into discrete wells in microtitre plates manually or by

using a bead dispenser. The protected cyclic peptide was released from the solid

15   support and deprotected in one step using a 95% TFA (5% cation scavengers)

solution. After cleavage, the solvent was removed *in vacuo* to yield cyclic

peptide with high purity. Cleavage conditions and work-up procedures were

generally optimized to eliminate non-volatile scavengers and potentially

deleterious side products from the final peptide. Peptide libraries obtained by

20   the above procedure were sufficiently pure for use in anti-microbial selection

assays.

Materials: Acetonitrile (HPLC grade), dichloromethane (optima grade),

dicyclohexylamine (DCHA), diethyl ether (anhydrous), dimethylformamide

(sequencing grade), diisopropylethylamine (DIPEA, peptide synthesis grade),

25   and piperidine (anhydrous) were purchased from Fisher and used without further

purification. Trifluoroacetic acid (TFA, New Jersey Halocarbon), and 2-(1-*H*-

benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU,

Novabiochem), benzotriazole 1-yl-oxy-tris-pyrrolidino-phosphonium

hexafluorophosphate (PyBOP, Novabiochem) were used without further

30   purification. Tetrakistriphenylphosphine palladium(0) was purchased from

Strem Chemicals. Commercially available *N*-Fmoc amino acids for solid-phase

peptide synthesis and trityl chloride PS (1% DVB, substitution 0.5 – 1.05 mmol

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g^{-1}) resin were used as obtained from Novabiochem or Bachem. Trityl chloride macrobead resin was obtained from Peptides International.

Prpeapartion of Fmoc-Lysine(Boc)-Oallyl. Fmoc-Lysine(Boc)-OAllyl was made according to the protocol of Kates, S. A.; Solé, N. A.; Johnson, C. R.;

5   Hudson, D.; Barany, G.; Albericio, F. *Tetrahedron Lett.* 1993, *34*, 1549–1552. Fmoc-Lys-(Boc)-OH (5 g, 10.6 mmol) was added to allyl bromide (25 mL, 0.29 mol), followed by DIPEA (3.73 mL). This mixture was heated at 90 °C for 1 h. The reaction was allowed to cool, concentrated by rotary evaporation, and after dilution with ethyl acetate was washed with 2 × 0.1 N HCl, 2 × saturated sodium

10   bicarbonate at pH < 9.5, followed by brine. The organic layer was filtered through a pad of silica gel and concentrated to afford a solid. This solid was washed with ether to provide a white powder that was used directly in the next step.

Deprotection of Side Chain Boc Protecting Group. An appropriate

15   amount of Fmoc-Lys(Boc)-OAllyl for a resin loading of 0.5 mmol g^{-1} was placed in a round bottom flask. Sufficient dichloromethane to dissolve the solid was added followed by an equivalent amount of TFA. After stirring for 1 h the solution was evaporated and the residue of Fmoc-Lys-OAllyl was dried *in vacuo.*

20   *Resin loading.* Trityl chloride resin was swollen in dry deacidified (Na_2CO_3) dichloromethane for 20 min. A solution of Fmoc-Lys-OAllyl in dichloromethane was added to the resin, immediately followed by 4 eq. of DIPEA. After shaking for 2 hours the resin was washed resin with dichloromethane, then shaken with 10% MeOH: 10% DIPEA: 80%

25   dichloromethane for 10 min. After washing with dichloromethane and drying *in vacuo* the resin loading was evaluated based on Fmoc released monitored by UV absorption at 290 nm.

Peptide Synthesis. Peptides were synthesized using standard solid-phase Fmoc protocols (see Wellings, D. A.; Atherton, E. *Methods Enzymol.* 1997, *289*,

30   44–67) on the Fmoc-Lys-OAllyl loaded trityl resin. Following synthesis of the linear peptide the resin was swollen in dry dichloromethane for 20 min. To the resin was added a degassed solution of 0.5 eq. $Pd(PPh_3)_4$ in 90% $CHCl_3$: 10% 4-

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methylmorpholine. After shaking under Ar for 5 hours the resin was washed

with a solution of 1% sodium dimethylthiocarbamic acid in DMF (3 × 2 min),

1% DIPEA (3 × 2 min) in DMF. After the final Fmoc deprotection (25%

piperidine in DMF, 2 × 10 min), the resin was washed thoroughly with DMF (3

5   × 3 min), 10% DIPEA/DMF (3 × 3 min), 0.8 M LiCl/DMF (3 × 3 min). The

resin was treated with 5 eq. PyBOP, 5 eq. HOAt, 20 eq. DIPEA in 0.8 M

LiCl/DMF for at least 12 hours. After washing with DMF (3 × 3 min), DCM (2

× 3 min) followed by MeOH the peptide was cleaved from the resin and

deprotected with 2.5% TIS: 2.5% H_2O: 95% TFA. Peptides were recovered by

10 precipitation with ether or by evaporation of the cleavage mixture.

Antimicrobial Assay

The antimicrobial activity of the peptides was determined using a broth

dilution assay essentially as descried in the guidelines of the National Committee

for the Control of Laboratory Standards (NCCLS) [National Committee for

15 Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility*

tests for bacteria that grow aerobically. Fourth edition. Approved Standard

(1997). Document M7-A4. (NCCLS, Villanova, Pennsylvania, 1997]. Test

tubes (macrodilution method) or microtiter plates (microdilution method)

containing two-fold serial dilution of peptides were inoculated with various

20 bacterial cultures. Controls included non-inoculated medium (sterility), vehicle

control, and various commercially available antibiotics for which minimal

inhibitory concentrations against tested organisms were known. *In vitro* results

using both methods were reproducible and in all cases MICs from the

macrodilution assays were equal to, or one dilution below, the inhibitory

25 concentrations determined by microdilution tests (*See:*V. Lorian, *Antibiotics in*

Laboratory Medicine, Williams and Wilkins, Baltimore 1991). The

microdilution method has the advantages of requiring less amount of peptide for

each assay and the possibility of multiple simultaneous inoculations and was

used in most studies. Several of the strains of bacteria tested are further

30 described in Table 3.

Table 3

Species	Strain	Type	Special Properties

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Species	Strain	Type	Special Properties
Bacillus cereus	ATCC 11778	Gram positive	
Enterococcus faecalis	ATCC 51299	Gram positive	Vancomycin resistant (vanB); resistant to vancomycin, gentamicin and streptomycin.
Enterococcus faecium	SP180	Gram positive	Vancomycin resistant (vanA); resistant to multiple antibiotics
Staphylococcus aureus	OS2	Gram positive	
Staphylococcus. aureus	ATCC 33591	Gram positive	Methicillin-resistant.
Staphylococcus. aureus	ATCC 33592	Gram positive	Methicillin-resistant; resistant to multiple antibiotics
Streptococcus pneumoniae	ATCC 6301	Gram positive	
Listeria monocytogenes	ATCC 19115	Gram positive	
Escherichia coli	K12	Gram negative	Reference strain
Escherichia coli	JM109 (DE3)	Gram negative	
Escherichia coli	EL744 K12 (tolC::kan)	Gram negative	Lacks key efflux pump component
Hemophilus. influenzae	ATCC 49247	Gram negative	Reference strain
Salmonella typhimurium	ATCC 14028	Gram negative	

MIC Determination; Broth Dilution Method

Preparation of peptide solutions. Stock peptide solutions were prepared in 5% DMSO in aq. sucrose (9%). Determination of peptide concentrations was

5 done by quantitative HPLC analysis using known concentrations of internal standards and/or by measuring UV absorption of tryptophane-containing peptide solutions in H_2O (λ=280 ε_{Trp}: 5690). Serial 2-fold dilutions were made in the above DMSO/aq. sucrose (9%) mixture with concentrations ranging approximately 400-2 µg/ml and aliquots were dispensed in test tubes (100 µl) for

10 the macrodilution test or in microtiter plates (20 µl) for the microdilution assays.

Inoculum preparation. Overnight cultures of different microorganisms grown in suitable media were diluted 4000 times to an approximate inoculum size of 2.5 x 10^5 cfu/ml.

Macrodilution method. Macrodilution methods were performed using

15 procedures similar to those described in the National Committee for Clinical

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Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically.* Fourth edition. Approved Standard (1997). Document M7-A4. (NCCLS,Villanova, Pennsylvania, 1997). Two ml aliquots of the above inoculum were dispensed to test tubes containing different peptide

5   solutions. After incubation at 37 °C with shaking for 18 hours the lowest concentration at which no bacterial growth was observed was recorded as the MIC.

Microdilution method. Microdilution methods were performed using procedures similar to those described in the National Committee for Clinical

10   Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically.* Fourth edition. Approved Standard (1997). Document M7-A4. (NCCLS,Villanova, Pennsylvania, 1997. Eighty µl aliquots of the above inoculum were dispensed into 96-well microtiter plates containing different peptide solutions and incubated for 18 h at 37 °C with shaking (plates

15   were sealed with parafilm to avoid excessive evaporation of culture medium) and MIC's were recorded. Each assay was performed at least twice and errors were typically ± one dilution.

MBC Determination

20   Minimum bactericidal concentrations (MBC) were determined according to the guidelines of the National Committee for the Control of Laboratory Standards (NCCLS). National Committee for Clinical Laboratory Standards. Methods for Determining Bactericidal Activity of Antimicrobial Agents. Approved Standard (1999). Document M26-A. (NCCLS, Villanova,

25   Pennsylvania, 1999). Fifty µl aliquots from MIC, 2xMIC and 4xMIC assay wells were removed and plated in antibiotic-free agar plates by using the lawning technique. Growth and sterility controls were sampled in the same manner. The lawned plates were incubated for 24-48 hours and the MBC was determined as the lowest concentration at which 99.9% killing was achieved.

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Membrane Depolarization Studies

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Membrane depolarization was monitored by a change in fluorescence emission intensity of the cyanine membrane potential-sensitive dye 3,3'-dipropylthiadicarbocyanide iodide (diSC3). Intact *S. aureus* were grown at 37 °C with agitation to mid log-phase (O.D.$_{600}$ = 0.5). Cells were centrifuged and

5   washed once with buffer (20 mM glucose, 5 mM HEPES pH 7.3) and resuspended to an O.D.$_{600}$ of 0.05 in a similar buffer containing 0.1 M KCl. Cells were incubated with 1 μM diSC$_3$ until a stable reduction of fluorescence was achieved (approximately 15 min), indicating incorporation of the dye into the bacterial membrane. Peptides were added from stock solutions (1 mg/ml)

10   dissolved in 5% DMSO in aqueous sucrose (9%) to achieve desired 0.1 to 10xMIC concentrations.

Hemolytic Sensitivity

Selectivity of cyclic peptides for bacterial over mammalian cells was

15   evaluated by measuring red blood cell hemolytic activities as described in Tosteson, M.T. Holmes, S.J., Razin, M. and Tosteson, D.C. Melittin Lysis of Red Cells, J. *Membrane Biol. 87*, 35-44(1985). Heparinized murine blood was centrifuged at 1000 x g for 10 minutes. The supernatant and the buffy coat were removed. Erythrocytes were washed three times with 0.9% saline solution and

20   then resuspended to a concentration of 5 % in saline containing 10% FBS (v/v). Red blood cells were then treated with serial dilutions of test peptides in a 96 well plate at 37 °C for 30 minutes. Control samples included a saline solution and 1 % Triton X-100 as 0 and 100 % hemolysis, respectively. In some cases, mellitin was used as a further control. Mellitin is a linear peptide that is

25   hemolytic against mammalian red blood cells *in vitro* at a concentration of about 10 µg/ml. Plates were centrifuged at 1000 g for 10 minutes. Aliquots of the supernatant were diluted 2 times with saline solution and the absorbance was measured at 560 nm.

30   **Preparation of Peptide Solutions for *In Vivo* Experiments**

Stock peptide solutions were prepared in aqeous sucrose (9%). To facilitate dissolution of the peptide, the initial suspension was sonicated for 15-

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20 min. The obtained solution was sterilized by passage through a sterile 0.45μm filter (COSTAR, μStar, Corning Inc.). Determination of peptide concentrations was done by UV absorption in H_2O (λ=280 ϵ_{Trp}: 5690 $cm^{-1}M^{-1}$ for tryptophan-containing peptides) of different aliquots and found to be 60-70%

5   of the one corresponding to w/v. Alternatively, the concentrations of various peptide stock solutions were determined by quantitative HPLC analysis using known concentrations of internal standards. Further the peptide solutions were appropriately diluted with sterile aq. sucrose (9%).

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Bacteria Preparation for *In Vivo* Protection Testing

Bacteria were prepared for in vivo using procedures similar to those as previously described. *See* V. Lorian, Antibiotics in Laboratory Medicine, Williams and Wilkins, Baltimore 1991. *S. aureus* MRSA bacteria (ATCC

5   33591) were grown at 37 °C in 5 mL of Antibiotic Medium-3 (AM-3, Difco Laboratories) with agitation for 12 hours to a stationary phase. Cells were collected by centrifugation, washed twice with saline solution and resuspended in about 10 mL of saline to an O.D.$_{650}$ of 1.2. This suspension was diluted ten times in a sterilized 5% mucin (Difco) in saline to a concentration of 2-

10   4×10^7 cfu/ml (actual inoculum size was verified by colony counts on agar plates).

Vancomycin resistant *Enterococcus faecium* (VREF) bacteria (ATCC 51575) were grown at 37 °C in 60 mL of Brain Heart Infusion medium (BHI, Difco Laboratories) with agitation for 16 hours to a stationary phase. Cells were collected by centrifugation, washed twice with saline solution and resuspended

15   in 6 mL of saline to a theoretical O.D.$_{650}$ of 9-10 (determined by taking OD.$_{650}$ of 10-20 fold diluted samples and correlating it to the original concentration). This suspension was diluted 15 times in a sterilized 5% mucin (Difco) in saline to a concentration of $5-7 \times 10^8$ cfu/ml (actual inoculum size was verified by colony counts on agar plates).

20

In Vivo Animal Studies

Peptides of the invention were tested in mice to ascertain whether they would protect the mice against bacterial infection using procedures similar to those described in N. Frimodt-M ller et al., *The Mouse Peritonitis/Sepsis Model*,

25   in Zak et al. (eds.) HANDBOOK OF ANIMAL MODELS OF INFECTION 127-37 (Academic Press 1999).

Male Balb-C mice (6 weeks old, approximately 20 g) were used in the study. *Staphylococcus aureus* MRSA bacteria (ATCC 33591) were grown at 37 °C with agitation for 12 hours to a stationary phase. Cells were collected by

30   centrifugation, washed twice with saline and resuspended to an O.D.$_{650}$ of 1.4. This suspension was diluted ten times with saline and resuspended to a concentration of $3-5 \times 10^7$ cfu/ml. For each peptide five groups of eight mice per

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group were infected I.P. with 0.5 ml of the above *Staphylococcus aureus* MRSA preparation (lethal dose). Forty-five minutes to an hour after infection, each group was treated with a different dose of peptide and a control group received vehicle only. Mice were monitored for fourteen days; death was defined as the

5   end point. Median protecting doses (PD_{50}) were calculated following the Reed and Muench method. *See* V. Lorian, Antibiotics in Laboratory Medicine, Williams and Wilkins, Baltimore 1991. For IV and SQ models peptide was administered immediately after IP infection.

10   **Toxicity Studies**

Peptides were tested for toxicity *in vivo* using procedures similar to those described previously. *See* V. Lorian, Antibiotics in Laboratory Medicine, Williams and Wilkins, Baltimore 1991. Mice (male Balb-C) in groups of 4-8 were given via IV, IP or SQ single bolucdoses of peptides and monitored for 14

15   days. Toxicity of sublethal doses was assessed based on the behavior and appearance of the mice after peptide administration compared to control mice receiving only vehicle. Signs of acute toxicity included lack of activity, red feet and tail, faster breathing. Death was defined as the end point for lethal doses of peptides.

20

Pathology Studies

Pathology effects of peptides cyclo[RRKWLWLW]-HCl and cyclo-[KQRWLWLW]-HCl were accessed with Balb-C (male 20-25 g). Peptides were administered IP at a lethal dose of 75 mg/kg in 9% sucrose, along with control

25   mice that received vehicle alone. After 50-60 min (in case of cyclo[KQRWLWLW]-HCl), and on the next day (in case of cyclo[RRKWLWLW]-HCl) mice were sacrificed and analyzed (Dr. Osborn, Vet. Pathologist, Department of Animal Resources, TSRI). Pathology studies included blood cell count, and histology examination of different tissues and

30   organs.

Toxicity of multiple dosing was also tested with peptide cyclo[KSKWLWLW]-HCl. Mice (male CD-1,Charles River labs, 20-25 g) were

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used for this study. A regiment of 200 mg/kg per day of the peptide solution in

9% sucrose for ten consecutive days was administered to 3 mice IP, along with 2

control mice that received vehicle alone. On day eleven mice were sacrificed

and analyzed (Dr. Osborn, Vet. Pathologist, Department of Animal Resources,

5   TSRI). Pathology studies included blood cell count, and histology examination

of different tissues and organs.

Pharmacokinetics

Pharmacokinetics were performed using methods similar to those

10   described previously. *See* V. Lorian, Antibiotics in Laboratory Medicine,

Williams and Wilkins, Baltimore 1991; W. A. Ritschel, G. L. Kearns Handbook

of Basic Pharmacokinetics, American Pharmaceutical Assoc. 5th edition,

Washington, DC 1999.

15   **Pharmacokinetics Studies Using c[RRKWLWLW]HCl**

IV injection: Solution of peptide in 9% sucrose (1 mg/mL) was injected

IV into the tail vein of Balb/C mice at a dose of 3.6 mg/kg. Then the blood was

collected immediately from one group of mice (3 mice per group) by bleeding

the tail, separately from each mouse (50-100 uL per mouse). Further collections

20   of blood were done from other groups of mice at times 20, 40, 70, 90, 120, 180,

and 260 min after injection. Plasma from each blood sample was separated

immediately after collection by spinning down red blood cells over a period of 5

min at 4000 rev./min. The plasma was then diluted with an equal volume of

saline and refrigerated until analysis. Storage of samples under these conditions

25   over a period of 1 month did not change the concentration of peptide.

IP injection: Solutions of peptide in 9% sucrose (8.1 mg/mL) were

injected IP into Balb/C mice at a dose of 100 mg/kg. Blood was collected

immediately from one group of mice (3 mice per group) by bleeding the tail,

separately from each mouse (50-100 uL per mouse). Further collections of blood

30   were done from other groups of mice at times 0.5, 1, 2, 4, 6, 10, 15 h after

injection. Plasma from each blood sample was separated immediately after

collection by spinning down red blood cells over a period of 5 min at 4000

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rev./min. Plasma was then diluted with equal volume of saline and refrigerated until analysis. Storage of samples under these conditions over a period of 1 month did not change the concentration of peptide.

Detection of cyclo[RRKWLWLW]-HCl *in plasma by HPLC:* Diluted
5   with saline plasma (50-100 uL) was added to an eluent A (0.1 % HCl in 99% H_2O/1% ACN (v/v)) (150-300 uL), vortexed, and partial precipitation was removed by centrifugation. The clear solution was injected into an HPLC and the peptide was detected at 280 nm, in the 8-10 min interval, using a gradient of eluent A (0.1 % HCl in 99% H_2O/1% ACN (v/v)) and eluent B (0.07 % HCl in
10   10% H_2O/90% ACN (v/v)) using a flow rate of 1.5 ml/min. The following gradient was used: 30 to 30 % B (5 min), followed by 30 to 37% B (5 min), followed by 37 to 40% B (12.5 min).

Pharmacokinetics Studies Using c[KSKWLWLW]HCl

15   *IV injection:* Solution of peptide in 9% sucrose (2 mg/mL) was injected IV into the tail vein of Balb/C mice at a dose of 5 mg/kg. Blood was then collected immediately from one group of mice (3 mice per group) by bleeding the tail, separately from each mouse (50-100 uL per mouse). Further collections of blood were done from other groups of mice at times 30, 60, 120, 230, and 300
20   min after injection. Plasma from each blood sample was separated immediately after collection by spinning down red blood cells over a period of 5 min at 4000 rev./min. Plasma was then diluted with an equal volume of saline and refrigerated until analysis. Storage of samples under these conditions over a period of 1 month did not change the concentration of peptide.

25   *IP injection:* Solution of peptide in 9% sucrose (9.8 mg/mL) was injected IP into Balb/C mice at a dose of 100 mg/kg. Blood was then collected immediately from one group of mice (3 mice per group) by bleeding the tail, separately from each mouse (50-100 uL per mouse). Further collections of blood were done from other groups of mice at times 0.5, 1, 2, 4, 6, 10 h after injection.
30   Plasma from each blood sample was separated immediately after collection by spinning down red blood cells over a period of 5 min at 4000 rev./min. Plasma was then diluted with an equal volume of saline and refrigerated until analysis.

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Storage at these conditions over a period of 1 month did not change the

concentration of peptide.

Detection of c($\underline{K}$S$\underline{K}$W$\underline{L}$W$\underline{L}$W) HCl *in plasma by HPLC:* Samples were

diluted with saline plasma (50-100 uL), added to an equal volume of eluent A

5   (0.1 % TFA in 96.5% H_2O/0.9% ACN/2.4% MeOH (v/v)), vortexed, and partial

precipitation was removed by centrifugation. The clear solution was injected into

HPLC and the peptide was detected at 280 nm, in the 18-20 min interval, using a

gradient of eluent A (0.1 % TFA in 96.5% H_2O/0.9% ACN/2.4% MeOH (v/v))

and eluent B (0.05 % TFA in 8% H_2O/72% ACN/20% MeOH (v/v)) using a

10   flow rate of 1.5 ml/min. The following gradient was used: 0 to 0 % B (5 min),

followed by 0 to 100% B (25 min).

Calculation of pharmacokinetic parameters: The area of HPLC peaks

corresponding to the peptide in analytical plasma runs was correlated to those of

the calibration injections with known amounts of peptide, and the determined

15   concentration was plotted against the time of blood collection. In the first

approximation the curve obtained from IV injection fits to the first order kinetic

equation (1), indicating a single compartment model. *See* V. Lorian, Antibiotics

in Laboratory Medicine, Williams and Wilkins, Baltimore 1991; W. A. Ritschel,

G. L. Kearns Handbook of Basic Pharmacokinetics, American Pharmaceutical

20   Assoc. 5th edition, Washington, DC 1999.

$$C_t = C_0 * e^{-Kel*t} \quad (1)$$

The best fit of equation (1) to the experimental points was calculated

25   using SigmaPlot. The following parameters were calculated from the best fit of

IV injection curve:

AUC_{IV} (μg*h/mL) - area under the curve for IV injections

C_0 (μg/mL) - concentration of peptide in blood at time zero

K_{el} (1/min) - elimination rate constant

30     $T_{1/2}$ (min) - half life time (time when C = C_0/2); $T_{1/2}$ = (ln2)/K_{el}

V (L/kg) - volume of distribution, V = D/C_0 , where D is the dose

of

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injected peptide expressed in mg/kg

CL (mL/min) - total clearance of peptide was calculated in two

ways and

averaged

5   CL = Dose/AUC$_{IV}$, where Dose is total dose of peptide received

by

mouse expressed in µg

CL = K$_{el}$ * V * m, where m is mass of the mouse expressed in g

10   Equation (2) was used to determine bioavailability (F) of peptide injected via IP

route

$$F = 100\%*(AUC_{IP} * D_{IV})/(AUC_{IV} * D_{IP}) \qquad (2)$$

15   where AUC$_{IP}$ is area under the concentration vs. time curve from IP injections,

D$_{IV}$ and D$_{IP}$ are doses of peptide administered via IV and IP routes respectively,

expressed in mg/kg.

Example 2.   *In Vitro* **Antibacterial Activity of Cyclic Peptides.**

20   The eight-residue cyclic peptides used in the first experiments of this

example had three consecutive polar residues and *L*-tryptophan and *D*-leucine

repeats that form a hydrophobic surface to promote effective partitioning onto

cell membranes. In addition, peptides were most often designed to include at

least one basic amino acid residue in order to enhance specificity toward

25   bacterial membranes.

The utility of these design features was demonstrated by *in vitro*

antibacterial assays employing a representative cyclic peptide with sequence

Lys-D-Gln-Arg-D-Trp-Leu-D-Trp-Leu-D-Trp (SEQ ID NO:9). Cyclic peptide

SEQ ID NO:9 displayed potent activity against gram positive *Bacillus subtilis*

30   and *Staphylococcus aureus* and against gram negative *Streptococcus*

pneumonieae and vancomycin-resistant *Enterococcus faecalis* (Table 4).

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Table 4. *In vitro* activity of Lys-D-Gln-Arg-D-Trp-Leu-D-Trp-Leu-D-Trp

(SEQ ID NO:9)

Gram positive bacteria	MIC[a] (μg/ml)
Bacillus subtilis [c]	3 [b]
Bacillus cereus [d]	2
Staphylococcus aureus [e]	3 [b]
Listeria monocytogenes [f]	20
Enterococcus faecalis [g]	10[b]
Streptococcus pneumoniea [h]	10[b]
Gram negative bacteria	**MIC (μg/ml)**
Salmonella typhimurium [i]	>70
Mammalian cells	
Red blood cell (mouse)	45 [j]
M21 (melanoma)	>75[k]

[a] Minimum inhibitory concentration;
[b] Data from MDS Panlabs Pharmacology Services;
[c] ATCC 43223;   [d] ATCC 11778;
[e] ATCC25923;   [f] ATCC19115;
[g] vancomycin-resistant clinical isolate;
[h] erythromycin- and ampicillin-resistant clinical isolate;
[i] ATCC 14028;
[j] Hemolytic dose where 50% of red blood cells are hemolyzed (HD$_{50}$
       (μg/ml))
[k] LD$_{50}$ (μg/ml).

A series of six- and eight-residue amphiphilic cyclic peptides were made to further probe the antibacterial activity of these peptides as antibacterial agents and to examine the relationship between surface properties, antibacterial activities, and membrane selectivity. Peptides were synthesized by standard solid-phase BOC or FMOC synthetic protocols, cyclized in solution or on solid support, purified by RP-HPLC, and characterized by MALDI-TOF or ESI-mass spectrometry. The sequences of these peptides are provided in Table 5. Shorthand representation of cyclic peptide sequences using single letter codes

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was used to allow facile sequence comparisons. Underlining indicates that amino acid is a D-amino acid residue.

Table 5: Peptide Sequences

SEQ ID NO:	Shorthand	Sequence Listing	Xaa Definition
1 – cyclic	QAQAQAQAQAQA	Gln-Xaa$_1$-Gln-Xaa$_1$-Gln-Xaa$_1$-Gln-Xaa$_1$-Gln-Xaa$_1$-Gln-Xaa$_1$	Xaa$_1$ = D-Ala
2 – cyclic	QLWLWLWL	Gln-Xaa$_1$-Trp-Xaa$_1$-Trp-Xaa$_1$-Trp-Xaa$_1$	Xaa$_1$ = D-Leu
3 – cyclic	KLWLWLWL	Lys-Xaa$_1$-Trp-Xaa$_1$-Trp-Xaa$_1$-Trp-Xaa$_1$	Xaa$_1$ = D-Leu
4 – cyclic	KLKLKLKL	Lys-Xaa$_1$-Lys-Xaa$_1$-Lys-Xaa$_1$-Lys-Xaa$_1$	Xaa$_1$ = D-Leu
5 – cyclic	SKSWLWLW	Xaa$_1$-Lys-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Ser Xaa$_2$ = D-Leu
6 – cyclic	THSWLWLW	Xaa$_1$-His-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Trp	Xaa$_1$ = D-Thr Xaa$_2$ = D-Ser Xaa$_3$ = D-Leu
7 – cyclic	RGDWLWLW	Xaa$_1$-Gly-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Trp	Xaa$_1$ = D-Arg Xaa$_2$ = D-Asp Xaa$_3$ = D-Leu
8 – cyclic	KQRWLWLW	Xaa$_1$-Gln-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Arg Xaa$_3$ = D-Leu
9 – cyclic	KQRWLWLW	Lys-Xaa$_1$-Arg-Xaa$_2$-Leu-Xaa$_2$-Leu-Xaa$_3$	Xaa$_1$ = D-Gln Xaa$_2$ = D-Trp
10 – cyclic	RQRWLWLW	Xaa$_1$-Gln-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Arg Xaa$_2$ = D-Leu
11 – cyclic	KQKWLWLW	Xaa$_1$-Gln-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
12 – cyclic	KSKWLWLW	Xaa$_1$-Ser-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
13 – cyclic	SHKWLWLW	Xaa$_1$-His-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Trp	Xaa$_1$ = D-Ser Xaa$_2$ = D-Lys Xaa$_3$ = D-Leu

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SEQ ID NO:	Shorthand	Sequence Listing	Xaa Definition
14 – cyclic	SKHWLWLW	Xaa$_1$-Lys-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Trp	Xaa$_1$ = D-Ser Xaa$_2$ = D-His Xaa$_3$ = D-Leu
15 – cyclic	SHHWLWLW	Xaa$_1$-His-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Trp	Xaa$_1$ = D-Ser Xaa$_2$ = D-His Xaa$_3$ = D-Leu
16 – cyclic	EKHWLWLW	Xaa$_1$-Lys-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Trp	Xaa$_1$ = D-Glu Xaa$_2$ = D-His Xaa$_3$ = D-Leu
17 – cyclic	KKKWLWLW	Xaa$_1$-Lys-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
18 – cyclic	RRKWLWLW	Xaa$_1$-Arg-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Trp	Xaa$_1$ = D-Arg Xaa$_2$ = D-Lys Xaa$_3$ = D-Leu
19 – cyclic	KRKWLWLW	Xaa$_1$-Arg-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
20 – cyclic	RRRWLWLW	Xaa$_1$-Arg-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Arg Xaa$_2$ = D-Leu
21 – cyclic	HKHWLWLW	Xaa$_1$-Lys-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-His Xaa$_2$ = D-Leu
22 – cyclic	KHKWLWLW	Xaa$_1$-His-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
23 – linear	WKKKWLWLW	Trp-Xaa$_1$-Lys-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
24 – linear	KKKWLWLW	Xaa$_1$-Lys-Xaa$_1$-Trp-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
25 – linear	Ac-KKKWLWLW-CONH$_2$	Xaa$_1$-Lys-Xaa$_2$-Trp-Xaa$_3$-Trp-Xaa$_3$-Xaa$_4$	Xaa$_1$=Ac-D-Lys Xaa$_2$ = D-Lys Xaa$_3$ = D-Leu Xaa$_4$ = D-Trp-CONH$_2$
26 – cyclic	KKLWLW	Xaa$_1$-Lys-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
27 – cyclic	KHLWLW	Xaa$_1$-His-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
28 – cyclic	KSLWLW	Xaa$_1$-Ser-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Lys Xaa$_2$ = D-Leu
29 – cyclic	RRLWLW	Xaa$_1$-Arg-Xaa$_2$-Trp-Xaa$_2$-Trp	Xaa$_1$ = D-Arg Xaa$_2$ = D-Leu

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SEQ ID NO:	Shorthand Sequence[c]	S. aureus (MRSA)[a] MIC µg/ml	E. coli[b] MIC µg/ml	Hemolysis HD50 µg/ml
14 – cyclic	SKHWLWLW	15	100	20
15 – cyclic	SHHWLWLW	20	100	20
16 – cyclic	EKHWLWLW	100	100	⟩100
17 – cyclic	KKKWLWLW	8	70	50
18 – cyclic	RRKWLWLW	6	15	50
19 – cyclic	KRKWLWLW	10	40	50
20 – cyclic	RRRWLWLW	10	50	35
21 – cyclic	HKHWLWLW	12	15	25
22 – cyclic	KHKWLWLW	10	80	30
23 - linear	WKKKWLWLW	60		
24 - linear	KKKWLWLW	50		
25 - linear	Ac-KKKWLWLW-CONH2	80		
26 – cyclic	KKLWLW	10	17	80
27 – cyclic	KHLWLW	10	100	25
28 – cyclic	KSLWLW	75	100	90
29 – cyclic	RRLWLW	35	5	90
30 – cyclic	QRUUUFWR[d]			
31 – cyclic	QRUUUWWR[d]			
32 – cyclic	QRUUUAWR		37	
33 – cyclic	QRUUULWR			
34 – cyclic	QRUUFWWR[d]	2.5 – 3.8		
35 – cyclic	QRUWLWWR[d]			

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SEQ ID NO:	Shorthand Sequence [c]	S. aureus (MRSA) [a] MIC μg/ml	E. coli [b] MIC μg/ml	Hemolysis HD50 μg/ml
36 – cyclic	QRWWLWW R[d]	2.5 – 3.8		
37 – cyclic	QRUUWWW R	2.5 – 3.8		
38 – cyclic	QRVWLWW R	2.5 – 3.8		
39 – cyclic	QRFWLWWR	2.5 – 3.8		
142 – cyclic	QRWWLWW R	2.5 – 3.8		

a. ATCC 33591. b. JM109(DE3). c. U is a hydrophobic amino acid selected from the group consisting of alanine, leucine, valine, phenylalanine and tryptophan. d. Activity against *Bacillus subtilis* at about 1.7 to 2.5 µg/ml. Peptide mixtures SEQ ID NO:30-37 are complex peptide mixtures and their concentrations for the anti-bacterial assay could not be specifically determined. The MIC values for these sequences may differ by up to five-fold.

The role of polar side chains in bioactivity and membrane selectivity may be evaluated upon examination of the antibacterial activities listed in Table 6.

Cyclic peptides with SEQ ID NO:5 and 6 each bear one basic residue between two serine residues, or between a serine and a threonine residue, respectively. While peptide SEQ ID NO:5 [cyclo-(D-Ser-Lys-D-Ser-Trp-D-Leu-Trp-D-Leu-Trp)] displays good activity against *S. aureus* MRSA, substitution of lysine by histidine in peptide SEQ ID NO:6 [cyclo-(D-Thr-His-D-Ser-Trp-D-Leu-Trp-D-Leu-Trp)] significantly decreases the activity.

Cyclic peptides with SEQ ID NO:8-15 each possess two basic amino acids and one neutral, polar amino acid. These cyclic peptides vary in antibacterial activity and in red blood cell hemolysis. A single glutamic acid substitution has a deleterious effect on the antibacterial activity as indicated by the large difference in activity against *S. aureus* MRSA bacteria between peptide SEQ ID NOs:14 [cyclo-(D-Ser-Lys-D-His-Trp-D-Leu-Trp-D-Leu-Trp)] and 16 [cyclo-(D-Glu-Lys-D-His-Trp-D-Leu-Trp-D-Leu-Trp)]. The reduced activity of the glutamic acid-containing peptide may be due to unfavorable electrostatic interactions of the carboxylate side chain with bacterial membrane constituents.

Increasing the number of basic residues from two to three in peptide SEQ ID NOs:17-22 provided high activities against *S. aureus* MRSA. Peptides with

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SEQ ID NO:18 [cyclo-(D-Arg-Arg-D-Lys-Trp-D-Leu-Trp-D-Leu-Trp)] and SEQ
ID NO:21 [cyclo-(D-His-Lys-D-His-Trp-D-Leu-Trp-D-Leu-Trp)] also exhibited
moderate activities against *E. coli*. The *in vitro* antibacterial activities of
hexameric peptides with SEQ ID NOs:26-29 also indicate that use of basic
5   amino acids in the cyclic peptides may increase antibacterial activity and
improve selectivity for bacterial membranes. Peptide SEQ ID NO:26 [cyclo-(D-
Lys-Lys-D-Leu-Trp-D-Leu-Trp)], with two consecutive lysine residues, exhibits
broad-spectrum activity and has low hemolytic properties. On the other hand,
peptide SEQ ID NO:27 [cyclo-(D-Lys-His-D-Leu-Trp-D-Leu-Trp)], which has a
10   histidine instead of the lysine in peptide SEQ ID NO:26, retains high activity
against *S. aureus* MRSA but is inactive against *E. coli*. Substituting a lysine in
peptide SEQ ID NO:26 with serine yields the less active peptide SEQ ID NO:28
[cyclo-(D-Lys-Ser-D-Leu-Trp-D-Leu-Trp)]. However, peptide SEQ ID NO:29
[cyclo-(D-Arg-Arg-D-Leu-Trp-D-Leu-Trp)], which possesses two arginine
15   residues, displays potent and selective activity against *E. coli* with low levels of
hemolysis.

The spectrum of activity and membrane selectivity observed with the
above peptides indicates that even single amino acid substitutions can influence
antibacterial activity and selectivity versus mammalian cells.

20   The effect of plasma proteins on the availability and stability of cyclic
peptides *in vitro* was also examined. Antibacterial activities remained
unchanged in the presence of large amounts (up to 50% v/v) of fetal bovine
serum (FBS) in the culture media. However, most peptides examined have a
reduced level of hemolytic activity in the presence of 5-10% FBS in the assay
25   mixture, compared to analogous assays in the absence of FBS. For example, in
the presence of 10% FBS, peptide SEQ ID NO:8 [cyclo-(D-Lys-Gln-D-Arg-Trp-
D-Leu-Trp-D-Leu-Trp)] displays five-fold reduction in hemolytic activity (from
HD_{50} = 10 to 50 μg/ml).

Cyclic peptides having SEQ ID NO:13, 18, 26, and 29 were assayed for
30   proteolytic susceptibility. The peptides show abiotic structure and
conformational preferences, and were stable in the presence of trypsin, α-
chymotrypsin, subtilisin, and blood plasma. No significant peptide degradation

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was observed in chromatograms obtained from RP-HPLC over a 24 h time period, whereas control linear L-α-amino acid peptides were degraded in less than 10 min under similar reaction conditions and within four hours when placed in murine blood plasma.

5   Results for further testing of gram-negative and gram-positive bacteria are shown in Tables 7 and 8 respectively, along with control assays using FDA approved antibiotics.

The shorthand representation of cyclic peptide sequences was utilized where single amino acid letter codes are employed to facilitate sequence comparisons.

10   Underlining indicates that amino acid is a D-amino acid residue and brackets indicate that the peptides are circular.

Table 7: Gram Negative Bacteria

Peptide Sequence	E. coli (K12) MHII broth	E. coli (EL744) K12 (tolC::kan) MHII broth	H. influenzae ATCC 49247 HTM
	MIC (ug/mL)	MIC (ug/mL)	MIC (ug/mL)
[KKLWLW]-HCl	>128	32	>128
[KSKWLWLW]-HCl	>128	>128	32
[RRLWLW]-HCl	16	8	8-16
[RRKWLWLW]-HCl	32	16	32-64
Kanamycin	4	>128	4
Norfloxacin	0.06	0.016	0.06
Penicillin G	32	16	4
Vancomycin	128	128	128

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Table 8: Gram-Positive Bacteria

Peptide Sequence	E. faecalis ATCC 51299 BHI broth	E. faecium SP180 BHI broth	S. aureus ATCC 33592 MHII broth
	MIC (ug/mL)	MIC (ug/mL)	MIC (ug/mL)
[KKLWLW]-HCl	>128	16	32
[KSKWLWLW]-HCl	8	4	4
[RRLWLW]-HCl	32-64	8-16	32

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[RRKWLWLW]-HCl	8-16	4	8
Kanamycin	>128	>128	>128
Norfloxacin	2	32	0.5
Penicillin G	0.5	128	64
Vancomycin	32	>128	0.5

The antibacterial properties of different types of cyclic peptides of the invention are further illustrated in Table 9. Each cyclic peptide is described in shorthand using underlining to indicate which amino acids have D-chirality, brackets to identify cyclic peptides and a number in parentheses to indicate the SEQ ID NO: (i.e., NO:). These peptides were tested against methicilin-resistant *Staphyococus aureus* (MRSA)(ATCC 33591) and vancomycin-resistant *Enterococus faecalis* (VRE)(ATCC 51575) and for hemolytic activity against mammalian red blood cells.

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Table 9: In Vitro Activity Against Methicilin-Resistant *Staphyococus aureus* (MRSA) and Vancomycin-Resistant *Enterococus faecalis* (VRE)

Peptide sequence	MRSA	VRE	RBC Hemolysis (HD$_{50}$)	Media Used
Control Antibiotics				
KANAMYCIN	150	>600	>600	b,c
MELLITIN	6.3	6.3	10	b,c
NORFLOXACIN	<2.8	<2.8	>360	b,c
VANCOMYCIN	<1.8	>240	>240	b,c
Group-I octamers				
[KFWLWLWF] (40)	33	>44	>44	a,a
[KLWLWLWL] (41)	12	25	30	b,c
Group-II octamers				
[KSWLWLWF] (42)	>26	>26	>26	a,a
[KWWLWLWH] (43)	13	17	>23	a,a
[KWWLWLKW] (44)	4	4	>58	a
Group-III octamers				
[EKHWLWLW] (16)	100	100	>100	a,a
[HKHWLWLW] (21)	12	16	25	a,a
[KHKLALAL] (45)	50	200	>200	b,c
[KHKLALWL] (46)	25	50	>200	b,c
[KHKLFLAL] (47)	3	13	>220	b,c
[KHKLFLFL] (48)	7	13	>220	b,c
[KHKLFLWL] (49)	12	25	200	b,c
[KHKLWLWL] (50)	9	18	150	b,c
[KHKWLWLW] (22)	10	10	30	a,a
[KKKFLFLF] (51)	18	75	>300	b,c
[KKKWLWLW] (17)	10	40	45	a,a
[KKKWLWLW-HCl] (17)	6	6	75	b,c
[KQRWLWLW] (9)	12	12	40	a,a
[KQRWLWLW-HCl] (8)	8	8	70	b,c
[KRKLFLFL] (52)	11	22	>180	b,c
[KRKWLWLW] (19)	15	8	50	a,a
[KSKLALAL] (53)	35	>280	>280	b,c
[KSKLFLFL] (54)	50	200	>200	b,c
[KSKLFLFL] (55)	200	50	>200	b,b
[KSKLGLGL] (56)	>240	>240	>240	b,c
[KSKLILIL] (57)	200	>200	>200	b,c

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Peptide sequence	MRSA	VRE	RBC Hemolysis (HD$_{50}$)	Media Used
[KSKLILIL] (58)	>200	50	>200	b,b
[KSKLNleLNleL] (59)	220	>200	>220	b,c
[KSKLVLVL] (60)	>220	>220	>220	b,c
[KSKLWLWL] (61)	15	15	125	b,c
[KSKLYLYL] (62)	100	200	>200	b,c
[KSKWLWLW] (12)	5	15	100	a,a
[KSKWLWLW-HCl] (12)	9	9	60	b,c
[KSOLWLWL] (63)	12	25	150	b,c
[KTKLALAL] (64)	80	>160	>160	b,c
[KTKLFLFL] (65)	60	>240	>240	b,c
[KTKLWLWL] (66)	18	18	150	b,c
[KWWLWLKS] (67)	7	10	>153	b,b
[QRFWLWWR] (68)	7.5	3.8	60	b,b
[QRLWLWWR] (69)	6.3	6.3	35	b,b
[QRVWLWWR] (70)	12.5	6.3	30	b,b
[QRWWLWWR] (71)	6.3	6.3	35	b,b
[RGDWLWLW] (7)	100	100	100	a
[RQRWLWLW] (10)	10	10	25	a
[RRKWLWLW] (18)	5	5	50	a
[RRKWLWLW-HCl] (18)	9	9	70	b,c
[SHHWLWLW] (15)	30	50	20	a
[SHHWLWLW-HCl] (15)	6	6	40	b,c
[SHKWLWLW] (13)	10	40	35	a,a
[SKHWLWLW] (14)	15	30	20	a,a
[SKSWLWLW] (5)	20	20	30	a,a
[THSWLWLW] (6)	100	100	100	a,a
Group-IV octamers				
[KHKHFLWL] (72)	8	16	200	b,c
[KHKHWLWL] (73)	18	18	150	b,c
[KRWLWLKH] (74)	5	7	>130	a,a
[KRWLWLKS] (75)	9	22	>48	a,a
[KSKKFLFL] (76)	5	10	100	b,c
[KSKKWLWL] (77)	13	27	>220	b,c
[KSSKALAL] (78)	>240	>240	>240	b,c
[KSSKFLFL] (79)	43	43	>200	b,c
[KSSKVLVL] (80)	22	180	>180	b,c
[KSSKWLWL] (82)	27	27	>220	b,c
[KSSKYLYL] (83)	85	170	>340	b,c
[KSWLWLRS] (84)	9	12	>62	a

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Peptide sequence	MRSA	VRE	RBC Hemolysis (HD$_{50}$)	Media Used
Group-V octamers				
[KHKHKWLW] (85)	6	6	150	b,c
[KHQHKLWL] (86)	100	100	>200	b,c
[KHQHKWLW] (87)	6	12	200	b,c
[KHSHKWLW] (88)	6	6	200	b,c
[KNQNKFWF] (89)	17	70	280	b,c
[KNQNKLWL] (90)	>200	>200	>200	b,c
[KSKSKWLW] (91)	6	12	200	b,c
[KSQSKWLW] (92)	9	18	195	b,c
[KSSSKWLW] (93)	6	6	175	b,c
[KYQYKNWN] (94)	>200	>200	>200	b,c
Group-VI octamers				
[KAQAKAWA] (95)	>200	>200	>200	b,c
[KAQNKAWN] (96)	>200	>200	>200	b,c
[KAQFKAWF] (97)	100	100	>200	b,c
[KFQFKNWN] (98)	>200	>200	>280	b,c
[KFWLWLHW] (99)	21	21	>25	a
[KFWLWLKF] (100)	11	10	>78	a
[KLKLKLKL] (3)	>200	>200	>200	b,c
[KRKWLHLW] (101)	40	40	100	a
[KRQFKFWF] (102)	12	12	>200	b,c
[KSKLHLHL] (103)	>320	>320	>320	b,c
[KSKLQLQL] (104)	>200	>200	>200	b,c
[KSKLRLRL] (105)	>240	>240	>240	b,c
[KSKLRLRL] (106)	>400	>400	>400	b,c
[KWKWSWLW] (107)	12	12	150	b,c
[KYQYKNWN] (108)	>200	>200	>200	b,c
[RRKWLELW] (109)	80	100	100	a
[RRKWLHLW] (110)	25	40	100	a
[RRKWLKLW] (111)	12	80	100	a
[RRKWLSLW] (112)	12	50	100	a
Group-II hexamers				
[KHLWLW] (27)	10	40	25	a
[KKLWLW] (26)	10	40	80	a
[KRVWAI] (113)	>200	>200	>200	b,c
[KSLWLW] (28)	100	100	90	b,c
[KWILAR] (114)	50	>200	>200	b,c
[RRWLWL] (115)	40	100	90	a,a
[KWQWLW] (116)	12	12	>16	a,a

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Peptide sequence	MRSA	VRE	RBC Hemolysis (HD$_{50}$)	Media Used
Group-III hexamers				
[KEKWLW] (117)	101	91	>121	a,a
[KEQWLW] (118)	>115	>115	>115	a,a
[KHKWLW] (119)	12	60	>115	a,a
[KHQWLW] (120)	43	57	115.0	a,a
[KKKWLW] (121)	23	51	>111	a,a
[KKQWLW] (122)	27	81	>215	a,a
[KNQWLW](123)	>74	>74	>74	a,a
[KQQWLW] (124)	>35	>35	>35	a,a
[KRKWLW] (125)	9	33	>174	a,a
[KSKWLW] (126)	19	38	>65	a,a
[KSYVWS] (127)	>200	>200	>200	b,c
Group-VI hexamers				
[KFKWLW] (128)	14	43	>61	a,a
[KFQWLW] (129)	25	10	>81	a,a
[KHQSRT] (130)	>200	>200	>200	b,c
[KHQSRT] (131)	>200	>200	>200	a,a
[KSIRIT] (132)	>200	>200	175	b,c

All peptides tested were TFA salts except where noted. Media "a" was anti-microbial media-3 (AM-3). Media "b" was cation adjusted Meuller Hinton broth (MHBII). Media "c" was Brain Heart Infusion (BHI).

5　　　　In another series of experiments (Table 10), the activity of cyclic peptides of the invention was tested against a larger variety of bacterial species. The bacterial species tested were as follows: Vancomycin resistant *Enterociccus faecalis* (VRE, ATCC 51575); methicilin resistant *Staphylococcus aureus* (ATCC 33591, MRSA); *E. coli*: JM109 (DE3) *Bacillus cereus* (ATCC11778);

10　　and *Streptococcus pneumoniae* (ATCC 6301). Murine red blood cells were used for the hemolysis assays as described in Example 1.

Table 10: Minimum Inhibitory Concentrations (MIC) in mg/ml

Peptide	VRE	MRSA	E. coli	B. cerius	S. pneum.	Hemolysis HD$_{50}$ mg/ml
Group-I octamer						
[KLWLWLWL] (3)	80	ppt	100	50	50	20
Group-II octamer						
[KELWLWLW] (143)	100	100		>140	100	

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Peptide	VRE	MRSA	E. coli	B. cerius	S. pneum.	Hemolysis HD$_{50}$ mg/ml
Group-III octamer						
[KSKWLWLW] (12)	15	5	40	<3	10	100
[RRKWLWLW] (18)	5	5	15	5	5	50
[RQRWLWLW] (10)	10	10	100	<1	4	25
[SHKWLWLW] (13)	40	10	100	12		35
[KKKWLWLW] (17)	40	10	65	5	10	45
[RRRWLWLW] (133)		10	35	10	5	35
[KHKWLWLW] (22)	10	10	80	8	8	30
[KQRWLWLW] (9)	12	12	80	3	4	40
[HKHWLWLW] (21)	16	12	15	4	19	25
[SKHWLWLW] (14)	30	15	100	6	5	20
[KRKWLWLW] (19)	8	15	60	3	5	50
[SKSWLWLW] (5)	20	20	100	>30	30	30
[KQKWLWLW] (11)	20	20	100	7	8	50
[SHHWLWLW] (15)	50	30	100	11	40	20
[THSWLWLW] (6)	100	100	100	>280	100	100
[RGDWLWLW] (7)	100	100	100	>280	70	100
[EKHWLWLW] (16)	100	100	100	85	50	>100
Group-VI octamer						
[RRKWLKLW] (111)	80	12	6	23	25	100
[RRKWLSLW] (112)	50	12	12	13	12	100
[RRKWLHLW] (110)	40	25	15	20	20	100
[KRKWLHLW] (101)	40	40	12	80	80	100
[RRKWLELW] (109)	100	80	40	195	100	100
linearized octamers						
H-WKKKWLWL-OH (144)	100	60		340	100	
H-KKKWLWLW-NH2 (134)	100	30	3	19	80	100
Ac-KKKWLWLW-NH2 (25)	100	100	20	75	80	100
H-RRKWLWLW-NH2 (135)	100	20	4	19	40	100
Ac-RRKWLWLW-NH2 (136)	100	70	6	22	40	100
H-LWRRKWLW-NH2 (137)	40	5	4	9	20	100
Ac-LWRRKWLW-NH2 (138)	100	15	7	20	20	100
Backbone modified octamers						
c[RRKW^{Me}LW^{Me}L W] (139)	30	5	5	5	10	100

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Peptide	VRE	MRSA	E. coli	B. cerius	S. pneum.	Hemolysis HD$_{50}$ mg/ml
c[KQRW^{Me}LW^{Me}L W] (140)	100	10		8	10	
Group-II hexamer						
[KKLWLW] (26)	40	10	17	>10	30	80
[KHLWLW] (27)	40	10	100	5	5	25
[RKLWLW] (141)	25	25		13	20	
[RRWLWL] (115)	100	40	5	24	12	90
[KSLWLW] (28)	100	100	100	15	20	90

The speed at which several of the cyclic peptides of the invention kill *E. coli* JM109 (DE3), *Enterococcus faecium* SP 180 and *S. aureus* (MRSA) (ATCC No. 33591 and 33592) was further tested at room temperature and/or at 37°C. A

5   lower temperature tends to enrich the bacterial cultures for mature bacteria because the bacteria are not growing quickly. Hence, at room temperature the culture will have more mature bacteria. The presence or absence of immature bacteria in the culture may influence the rate at which the bacteria are killed, because immature bacteria tend to be more susceptible to many forms of

10   antibiotics.

Cyclic peptides having the following sequences were used for testing.

D-Lys-His- D-Leu-Trp- D-Leu-Trp (SEQ ID NO:27);

D-Lys-Arg- D-Lys-Trp- D-Leu-Trp- D-Leu-Trp (SEQ ID NO:19);

D-Lys-Gln- D-Arg-Trp- D-Leu-Trp- D-Leu-Trp (SEQ ID NO:8);

15   D-Lys-Lys- D-Leu-Trp- D-Leu-Trp (SEQ ID NO:26);

D-Arg-Arg- D-Leu-Trp- D-Leu-Trp (SEQ ID NO:29);

D-Lys-Ser- D-Lys-Trp- D-Leu-Trp- D-Leu-Trp (SEQ ID NO:12);

and

D-Arg-Arg- D-Lys-Trp- D-Leu-Trp- D-Leu-Trp (SEQ ID NO:18).

20   Time curves for killing various types of bacteria are provided in Figures 8-20.

To generate the data shown in Figure 8a, the log of the number of colony forming units (cfu) per ml of culture was determined as a function of time after treatment of *S. aureus* (MRSA) (ATCC No. 33591) with cyclo[KHLWLW] (closed circles), cyclo[KRKWLWLW] (closed triangles), and

25   cyclo[KQRWLWLW] (open squares) at MIC concentrations. As illustrated in Figure 8a, cyclic peptides cyclo[KRKWLWLW] and cyclo[KQRWLWLW]

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killed effectively substantially all or most of the *S. aureus* (MRSA) within about

five minutes. The reduction in cfu count was greater than 3 log units. Cyclic

peptide cyclo[KHLWLW] killed effectively all *S. aureus* (MRSA) within about

60 minutes.

5   In Figure 8b, the log of the number of *E. coli* colony forming units (cfu)

per ml of culture is plotted versus time treated with cyclo[KRKWLWLW]

(closed triangles), cyclo[KQRWLWLW] (closed circles), cyclo[KHLWLW]

(closed squares), and cyclo[KKLWLW] (open squares) at MIC concentrations.

As illustrated, cyclo[KQRWLWLW] kills effectively all the *E. coli* within about

10   thirty minutes. Cyclic peptide cyclo[KHLWLW] kills effectively all *E. coli*

within about 90 minutes. Cyclic peptides cyclo[KRKWLWLW] and

cyclo[KKLWLW] kill effectively all of the *E. coli* within about 130 minutes. A

comparison of Figures 8 and 9 indicates that these cyclic peptides kill gram-

positive bacteria like *S. aureus* (MRSA) (ATCC No. 33592) somewhat faster

15   than they kill gram-negative bacteria like *E. coli*. Nonetheless, these cyclic

peptides kill both types of bacteria very quickly.

           Figures 9 and 10 illustrate the time course for killing *S. aureus* (MRSA)

(ATCC No. 33592) with varying concentrations of cyclo[KSKWLWLW] cyclic

peptide (SEQ ID NO:12) at room temperature and at 37° C. For example, at 37°

20   C, about 4 µg/ml of the KSKWLWLW cyclic peptide appears to kill effectively

all bacteria within about one hour. However, the bacterial population rebounds

by about six hours. This may be due to growth of a few survivors after the

residual cyclic peptide within the culture has been exhausted because the amount

of peptide employed in that assay was below the MIC value. At room

25   temperature, about 4 µg/ml appears to kill all *S. aureus* (MRSA) by about 2

hours and no rebound is observed. The KSKWLWLW cyclic peptide is highly

effective against bacteria at room temperature and at 37° C.

           Figures 11 and 12 illustrate the time course for killing *E. faecium* SP180

with varying concentrations of KSKWLWLW (SEQ ID NO:12), at room

30   temperature and at 37° C. Assays were performed in Brain Heart Infusion broth.

As illustrated, somewhat shorter times and/or somewhat lower concentrations of

this cyclic peptide are required to kill *E. faecium* SP180 at 37° C than at room

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temperature. *E. faecium* SP180 is vancomycin resistant (vanA) and multiply resistant to several FDA approved antibiotics. For example, at 37° C, about 2 μg/ml of K̲S̲K̲W̲L̲W̲L̲W can kill effectively all bacteria faster than an aliquot can be removed from the test culture for assessing the number of surviving cfu.

5   However, at room temperature, discernible numbers of surviving bacteria can be detected immediately after adding about 2 μg/ml of K̲S̲K̲W̲L̲W̲L̲W. Moreover, when 1μg/ml of K̲S̲K̲W̲L̲W̲L̲W is used, effectively no cfu can be detected after 2 hours at 37°C, whereas at room temperature, viable bacteria can be detected throughout the entire time-course. There is a rebound in bacterial viability when

10   using 1 μg/ml of KSKWLWLW at 37°C. This may be due to growth of a few survivors after the residual cyclic peptide within the culture has been exhausted because the amount of peptide employed in that assay was below the MIC value. The KSKWLWLW cyclic peptide is highly effective against *E. faecium* SP180 bacteria at both room temperature and at 37°C.

15   Figures 13 and 14 illustrate the time course for killing *S. aureus* (MRSA) (ATCC No. 33592) with varying concentrations of R̲R̲K̲W̲L̲W̲L̲W (SEQ ID NO:18), at room temperature and at 37° C. For example, at 37° C, about 4 μg/ml of the R̲R̲K̲W̲L̲W̲L̲W (SEQ ID NO:18) cyclic peptide appears to kill effectively all bacteria within about one hour. However, the bacterial population

20   rebounds by about eight hours. This may be due to growth of a few survivors after the residual cyclic peptide within the culture has been exhausted because the amount of peptide employed in that assay was below the MIC value. At room temperature, about 4 μg/ml appears to kill all *S. aureus* (MRSA) by about 1 hour after exposure to the cyclic peptide, and no rebound is observed. The

25   R̲R̲K̲W̲L̲W̲L̲W (SEQ ID NO:18) cyclic peptide is highly effective against bacteria at room temperature and at 37° C.

        Figures 15 and 16 illustrate the time course for killing *E. faecium* SP180 with varying concentrations of R̲R̲K̲W̲L̲W̲L̲W (SEQ ID NO:18) at room temperature and at 37° C. *E. faecium* SP180 is vancomycin resistant (vanA) and

30   multiply resistant to several FDA approved antibiotics. Assays were performed in Brain Heart Infusion broth. As illustrated, somewhat shorter times and/or somewhat lower concentrations of this cyclic peptide are required to kill *E.*

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faecium SP180 at 37° C than at room temperature. For example, at both room temperature and at 37° C, about 2 μg/ml of R̲R̲K̲WL̲WL̲W (SEQ ID NO:18) can kill effectively all bacteria within one hour. Hence, the R̲R̲K̲WL̲WL̲W (SEQ ID NO:18) cyclic peptide is highly effective against *E. faecium* SP180

5   bacteria at both room temperature and at 37°C.

Many conventional antibiotics are merely bacteriostatic, meaning they stop bacteria from growing but do not kill bacteria quickly. This is illustrated in Figures 17 and 18, which provide a time course for killing *S. aureus* (MRSA) (ATCC No. 33592) with varying concentrations of penicillin G. As illustrated,

10   at room temperature no concentration of penicillin G assayed up to 512 μg/ml was effective for killing, or even reducing, the entire population of *S. aureus* (MRSA) during the entire time course of the study (0-8 hours). However, at the highest concentration employed (512 μg/ml) penicillin G was effective in reducing the number of cfu within four hours at 37 °C. In contrast, as shown in

15   Figures 8-16, cyclic peptides of the invention quickly kill a variety of mature and immature bacteria at comparatively low concentrations.

Example 3.   Membrane Depolarization Activity.

Although not intending to be bound by any mechanism of action, it is

20   believed that one mechanism by which the present cyclic peptides may operate to kill microbes is by membrane depolarization. Another mechanism by which the cyclic peptides may kill microbes is through a receptor that recognizes the cyclic peptides as ligands. *See* Friederich et al., *Antimicrob. Agents Chemother.* 44, 2086-2092(2000); Amsterdam, D. in *Antibiotics in Laboratory Medicine,* 3rd

25   ed. (ed. Lorian, V.) 53-105 (Baltimore, Maryland, USA, 1991). A receptor/ligand-mediated mode of action for the cyclic peptides in bacterial membranes is unlikely for several reasons. First, cyclic peptides with diverse structures are active against microbes, but most receptors discriminate between potential ligands on the basis of structure, and will only recognize ligands having

30   defined structural features. Second, the peptides of the invention kill bacteria very quickly whereas a receptor/ligand-mediated binding/inhibition mechanism would typically be expected to take several hours to achieve complete

bactericidal or bacteristatic activity. This example provides biophysical data that supports a membrane depolarization mechanism of action.

The structural diversity of cyclic peptides with activity is illustrated by enantiomeric peptides having SEQ ID NO:8 (cyclo-[D-Lys-Gln-D-Arg-Trp-D-
5   Leu-Trp-D-Leu-Trp]) and SEQ ID NO:9 (cyclo-[Lys-D-Gln-Arg-D-Trp-Leu-D-Trp-Leu-D-Trp]). These two cyclic peptides have similar *in vitro* activities despite the differences in chirality of these peptides at each position. Moreover, as illustrated by the time-killing studies described in the previous Example, the speed of bacteria killing is not consistent with a receptor/ligand-mediated mode
10   of action. Such a receptor-ligand mechanism would likely require more time to kill bacteria than the short time periods shown in Figures 8-16. For example, octameric peptides having SEQ ID NO:8 and SEQ ID NO:19, and hexameric peptides having SEQ ID NO:26 and SEQ ID NO:27 at concentrations equal to or above the MIC have complete bactericidal activity against *S. aureus* MRSA
15   within 5 and 60 minutes, respectively. Further examples of time-killing studies are provided in the previous Example.

In this Example, cyclic peptides were tested to determine whether they would exhibit cellular depolarization activity. The fluorescence of the cyanine membrane dye 3,3'-dipropylthiadicarbocyanide is sensitive to changes in
20   bacterial membrane potential, and was employed in this Example to follow the effects of peptide SEQ ID NO:8, 18 and 26 on membrane depolarization of live *S. aureus* (ATCC 25923). *See,* Sims et al., *Biochemistry* 13, 3315-3330 (1974); Waggoner, *Annu. Rev. Biophys. Bioeng.* 8, 847-868 (1979); Loew, *Adv. Chem. Ser.* 235 (Biomembrane Electrochemistry), 151-173 (1994).

25   In each case, the exposure of dye-saturated live *S. aureus* to cyclic peptides at various concentrations (0.1 to 1 x MIC, minimum inhibitory concentration) led to fast and complete membrane depolarization (Figure 3). Culture samples taken during the course of these experiments correlate membrane depolarization with cell death. Substantially no living bacteria could
30   be detected after five minutes exposure to MIC concentrations of peptides, whereas at 0.1xMIC concentrations a significant population of live bacteria was still present.

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Biophysical Studies in Synthetic Membranes

The biophysical analyses performed in synthetic lipid membranes also support a membrane permeation mode of action. Studies support the idea that

5   eight residue cyclic D, L-α-peptides can likely form different types of supramolecular structures depending on the amino acid composition and sequence of the cyclic peptide. For example, the peptide SEQ ID NO:2 (cyclo[-Gln-D-Leu-(Trp-D-Leu)$_3$]) facilitates only the transport of analytes that are smaller than its internal diameter across liposome membranes and according to

10   ATR-FTIR analysis in DMPC multibilayes, assembles into a tube-like structure that is oriented perpendicular to the membrane plane. Therefore, the single nanotube through-pore mechanism it is a likely mode of function for this peptide (see Figure 2a).

On the other hand, the homologous peptide SEQ ID NO:3 (cyclo[-Lys-

15   D-Leu-(Trp-D-Leu)$_3$]), having a polar charged side chain likely forms a different supramolecular structure with an opening that is larger than the internal tube diameter because it facilitates transport of larger molecules of up to approximately 10,000 MW across membranes. Furthermore, the supramolecular structure formed from peptides having SEQ ID NO:3 (cyclo[-Lys-D-Leu-(Trp-

20   D-Leu)$_3$]) maintains an orientation in synthetic membranes that is perpendicular to the membrane plane according to ATR-FTIR analysis. These data support the conclusion that the peptide likely forms a barrel-stave type supramolecular structure in which multiple upright nanotubes bundle to form a larger pore opening (Figure 2b).

25   In comparison, peptides like SEQ ID NO:11 (cyclo[-(D-Lys-Gln-D-Lys-(Trp-D-Leu)$_2$-Trp-], SEQ ID NO:8 (cyclo[-(D-Lys-Gln-D-Arg-(Trp-D-Leu)$_2$-Trp-], SEQ ID NO:17 (cyclo[-(D-Lys-Lys-D-Lys-(Trp-D-Leu)$_2$-Trp-], and SEQ ID NO:18 (cyclo[-(D-Arg-Arg-D-Lys-(Trp-D-Leu)$_2$-Trp-] which possess a hydrophilic face of three contiguous residues, not only mediate the transport of

30   molecules larger than the internal peptide diameter (Figure 4), but also adopt orientations that are approximately parallel to the plane of the membrane structure. The ATR FT-IR spectroscopy of cyclic peptide SEQ ID NOs:8, 17

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and 18 in synthetic lipid membranes reveal amide-I and amide-II bands that are characteristic of tightly hydrogen bonded β-sheet-like structures (Table 11). The observed amide-A (NH stretch) frequencies support a tight network of intersubunit backbone hydrogen bonding. Quantitative measurements in

5 oriented DMPC lipid multibilayers indicate that self-assembled peptide nanotubes are oriented at a 70±5° tilt angle from the membrane normal. These observations suggest a carpet-like mode of membrane permeation (Figure 2). Increasing the hydrophilicity of the cyclic peptide, thus appears to favor a "surface-seeking" orientation over a "pore-forming" orientation.

10

Table 11. ATR-FTIR data and orientation of antibacterial cyclic peptide nanotubes in oriented DMPC lipid multibilayers.

Cyclic Peptide SEQ ID NO:	Frequency (cm^{-1})			Peptide		Lipid		ΔTilt[d]
	Amide -I	Amide -II	Amide -A	$[A\|\| / A^\wedge]$[a]	Tilt[c]	$[A\|\| / A^\wedge]$[b]	Tilt[c]	
8 [KQRWLWLW]	1629	1538	3283	1.16	70°	1.16	29°	41°
17 [KKKWLWLW]	1629	1537	3281	1.13	71°	1.17	29°	42°
18: [RRKWLWLW]	1628	1538	3280	1.34	65°	1.15	29°	36°

[a] The dichroic ratio of the amide-I intensity with parallel polarized incident light
15 to the band intensity with the perpendicular polarized light.
[b] The dichroic ratio of the antisymmetric CH_2 stretches of DMPC lipid.
[c] Tilts refer to the angle of the molecular axis with respect to the surface normal.
[d] Difference between the angles of the peptide tube axis and the lipid hydrocarbon chain. Lipid and peptide nanotube tilt angles are calculated
20 according to methods detailed in H.-S. Kim et al., 120 J. Am. Chem. Soc. 4417-24 (1998). DMPC: dimyristoyl phosphatidylcholine. Data are average of two samples with errors <±2°.

Further evidence for bacterial cellular membrane activity is provided by
25 electron micrographs of bacteria treated with cyclic peptides of the invention. Bacteria (*S. aureus*, ATCC 25923) were treated with cyclic peptide cyclo[KSKWLWLW] for 120 minutes at room temperature and then prepared for thin section electron microscopy by standard procedures.

Figures 21-23 illustrate the membrane effects caused by cyclic peptides
30 of the invention as observed using the electron microscope. Figure 21 provides a thin section electron microscopy image of untreated *S. aureus* (ATCC 25923)

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displaying a normal intact membrane. Figures 22 and 23 provide thin section electron microscopy images of *S. aureus* (ATCC 25923) after exposure to 2XMIC concentrations of cyclo[KSKWLWLW]. These images provide direct visualization of a membrane mode of action. Arrows denote abnormal

5 membrane structures caused by the peptide action.

Evidence thus indicates an anti-microbial activity based at least in part on membrane permeation, depolarization, and/or lysis. Such evidence includes that the cyclic peptides act very quickly to kill microbes, that diverse cyclic peptide structures described here show anti-microbial activity, that the cyclic peptides

10 can depolarize microbial membranes, that attenuated total reflectance (ATR) FT-IR spectroscopy studies are consistent with a membrane permeation mechanism of action rather than a receptor/ligand-mediated binding/inhibition mechanism, and that electron microscopy reveals an effect of the cyclic peptides of the invention on membrane structure.

15

Example 4.   Anti-microbial Activity *In Vivo*.

Toxicology Studies: Initial toxicology studies in mice were conducted to evaluate various routes of drug administration, maximum tolerable dose, and blood and tissue toxicity. Two mice in each dose study were injected with a

20 bolus intravenous (IV), intraperitoneal (IP), or subcutaneous (SQ) doses of peptide SEQ ID NO:8, 18, and 26. For each study, two control animals received vehicle only and no peptide. Studies with peptide SEQ ID NO:8 indicate that bolus IV injections of 12 mg/kg cause signs of temporary (<10 min in duration) discomfort. In contrast, no signs of toxicity were observed at the maximum

25 tested dose (12 mg/kg) with the IP route of administration. Peptide SEQ ID NO:26 was tolerated up to the maximum tested IP dose (50 mg/kg), with the highest dose causing signs of acute toxicity that were not observable after one hour. Signs of acute toxicity included any one of the following: lack of activity, red feet and tail, or faster breathing. Peptide SEQ ID NO:18 was tested up to

30 17.5 mg/kg IP and SQ doses without any apparent signs of toxicity. A regimen of 17.5 mg per day of peptide SEQ ID NO:18 for three consecutive days was administered to two mice IP and two mice SQ. Over four days, no apparent

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changes in the physical, social, and feeding activities were observed in these mice relative to control mice injected with vehicle without peptide. Moreover, hematology, necropsy and detailed microscopic examination of various tissue and organ samples performed on the fourth day after the initial injection showed

5   normal blood and morphological profiles except for the sites of IP and SQ injections (K.G. Osborn, DVM, Ph.D., the Scripps Research Institute). These sites exhibited moderate subacute inflammation typical of IP and SQ drug administration.

      In Vivo Antibacterial Effects: Peptide SEQ ID NO:18 was tested for

10   antibacterial *in vivo* by observing whether this peptide could protect mice from bacterial infection. Two groups of mice (4 mice per dose in each group) were infected intraperitoneally (left side) with a lethal dose of MRSA (ATCC 33591) ($2\text{-}5 \times 10^7$ cfu/mouse). To the first group of mice, peptide bolus doses of 0 (vehicle only), 10, 20, and 40 mg/kg were administered subcutaneously (SQ) in

15   the upper neck compartment soon after MRSA injection. To the second group of mice, five bolus peptide doses each 0 (vehicle only), 2.5, and 5 mg/kg were administered intravenously (IV) in 10 hour intervals with the first series of doses administered soon after MRSA injection. All mice in the control groups that received vehicle without peptide (0 mg/kg doses) died within 48 hours.

20   However, at 40 mg/kg bolus SQ dose and 2.5 x 5 mg/kg IV dosage regimen, 75% and 50% of mice survived, respectively, during the course of a fourteen-day study (Figure 28).

      In general, some variability was observed in the data for SQ and IV treatments. Such variability may result from a low absorption of the peptide

25   administered SQ and/or due to the range of expected experimental errors in IV administration of drugs to mice, a small test animal. Therefore, we examined the efficacy of intraperitoneal (IP) route of treatment. Six mice were infected intraperitoneally (left side) with a lethal dose of MRSA ($2\text{-}5 \times 10^7$ cfu/mouse). A 13 mg/kg bolus of the peptide was administered IP (right side) 45 min to an hour

30   after MRSA injection. All mice in the control group that received vehicle without peptide died within 48 hours. However, 67% of mice that received the peptide survived during the course of a seven-day study.

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Following this initial demonstration of *in vivo* antibacterial efficacy, a larger study with peptides having SEQ ID NO: 8, 12, 17, 18, and 26 was performed. Groups of mice were infected with lethal doses of MRSA (ATCC 33591) (IP left side). Each group of mice was treated with a bolus IP (right side)

5   dose of peptide SEQ ID NO: 8, 12, 17, 18, or 26 at 45-60 min after initial infection. The mice were observed for up to 14 days. All mice in the control group that received vehicle without peptide died within 48 hours. In each case, a single dose of the appropriate amount of peptide was sufficient to completely protect various groups of mice from MRSA infections (Figures 6, 24, 25, and 26;

10   Table 12).

Groups of mice were also infected with lethal doses of VREF (ATCC 51575) (IP left side). Each group of mice was treated with a bolus IP (right side) dose of peptide SEQ ID NO: 12, 17, or 18 at 45-60 min after initial infection. The mice were observed for 14 days. All mice in the control group that received

15   vehicle without peptide died within 48 hours. In each case, a single dose of the appropriate amount of peptide was sufficient to completely protect various groups of mice from VFEF infections (Figure 27, Table 12).

Table 12: *In vivo* Protection Doses (PD$_{50}$) and Lethal Doses (LD$_{50}$) of cyclic

20   **peptides**

Sequence[a]	PD$_{50}$ (μg/ml)		LD$_{50}$(IP) (μg/ml)	LD$_{50}$(IV) (μg/ml)
	MRSA[b]	VREF[c]		
c[RR**K**WL**W**L**W**] (SEQ ID NO:18)	8	7	60	16
c[**KQ**RWL**W**L**W**] (SEQ ID NO:8)	7	-	55	>12
c[**KS**KWL**W**L**W**] (SEQ ID NO:12)	20	5	>400	70
c[**KKK**WL**W**L**W**] (SEQ ID NO:17)	7	13	-	-
c[**KKL**WL**W**] (SEQ ID NO:26)	15	-	75	24

[a] Underlined letters represent D-residues. [b] MRSA (ATCC 33591). [c] VREF (ATCC 51575). Male Balb- C mice were used in groups of 8 (for MRSA) and 4 (for VREF and lethal doses).

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Remarkably, the effective doses were parallel to those required to kill bacteria *in vitro*. Specifically, the dosages of peptides having SEQ ID NO:18, 8, 12, 17, and 26 at which 50% of animals are protected from *S. aureus* MRSA infection (PD_{50}) were 8 mg/kg, 7 mg/kg, 20 mg/kg, 7 mg/kg, and 15 mg/kg,

5   respectively. Similarly, the dosages of peptides having SEQ ID NO:18, 12 and 17 at which 50% of animals are protected from *E. faecium* VREF infection (PD $_{50}$) were 7 mg/kg, 5 mg/kg and 13 mg/kg, respectively. The trend of these values is similar to the trend of the MIC values observed for *in vitro* antibacterial studies, as shown by Table 13, below.

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Table 13: Comparison of Effective Amounts In Vitro and In Vivo

Peptide	*In Vitro*			*In Vivo*		
	MRSA MIC (µg/mL)	*VREF* MIC (µg/mL)	*RBC* MIC (µg/mL)	*MRSA* PD_{50} mg/kg	*VREF* PD_{50} mg/kg	LD_{50}(ip) mg/kg
c[RRK WLWL W] (SEQ ID NO:18)	6	5	50	8	7	60
c[KQR WLWL W] (SEQ ID NO:8)	12	12	40	7	-	55
c[KSK WLWL W] (SEQ ID NO:12)	5	15	100	20	5	>400
c[KKL WLW] (SEQ ID NO:26)	10	40	80	15	-	75

These *in vivo* studies support the use of the cyclic peptides of the present

15   invention as anti-microbial agents, including use of agents in the treatment of multiply drug-resistant *Staphylococcus aureus, Enterococcus feacium* and other bacterial infections.

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Pharmacokinetic Studies: Pharmacokinetic parameters for peptides cyclo[RRKWLWLW] and cyclo[KSKWLWLW] in mice and for commercial antibiotics in humans are provided in Table 14.

Table 14: Pharmacokinetic Comparisons

Pharmacokinetic Parameter	c[RRKWLWLW] Mouse	c[KSKWLWLW] Mouse	Vancomycin[a] Human	Erythromycin[a] Human
K_{el} (1/h)	0.6	0.84	0.12	0.58
V_D (L/kg)	0.58	0.67	0.47	-
$T_{1/2}$ (h)	1.20	0.83	6	1.2
CL (mL/min)	0.16	0.30	66	-
F_{IP} (%)	9	32	-	-

K_{el} – elimination rate constant, V_D – volume of distribution, $T_{1/2}$ – half life time, CL – total clearance, F_{IP} – bioavailability via IP route. a) from David Bourne, Ph.D., www.boomer.org/c/p1/

Example 5. Cyclic β-Peptides Self-Assemble to Form Transmembrane Ion Channels

The ion transport properties of tetrapeptides cyclo[(β-Trp)$_4$] and cyclo[(β-Trp-β-Leu)$_2$], synthesized via established protocols, were studied in liposome-based assays and by single channel conductance measurements in planar lipid membranes. The observed conductance in 500 mM KCl with peptide concentrations of about 30 mM in the subphase is 56 pS, corresponding to the rates of channel mediated K^+ transport of 1.9×10^7 ions s^{-1} for both tetrapeptides. Such a transport speed is more than twice that of gramicidin A under similar conditions.

FT-IR studies in lipid membranes were also undertaken that provided evidence of transmembrane channels formed by these peptides. Peptide preparations displayed all expected peptide IR signals including amide I, amide II and N-H bands. The observed amide N-H stretching bands at 3289 cm^{-1} and 3297 cm^{-1} indicate the existence of tight backbone hydrogen-bonding networks

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with an average inter-subunit distance of 4.8 Å that are consistent with solid

state IR data on cyclic D, L-α-peptides. Therefore, cyclic β-peptides can also

form transmembrane ion channels.

All publications and patents are incorporated by reference herein, as

5   though individually incorporated by reference. The invention is not limited to

the exact details shown and described, for it should be understood that many

variations and modifications may be made while remaining within the spirit and

scope of the invention defined by the statements.

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WHAT IS CLAIMED:

1. A pharmaceutical composition for treating or preventing a microbial infection in an animal comprising a pharmaceutically acceptable carrier and a cyclic peptide having a sequence of from four to about sixteen alternating D- and L-α-amino acids in an amount effective to treat or prevent said infection caused by a target microbial organism in said animal.

2. The pharmaceutical composition of claim 1, wherein the cyclic peptide has a minimum inhibitory concentration at which substantially no target microbial organisms grow *in vitro* that is less than about one half the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells *in vitro*.

3. The pharmaceutical composition of claim 1, wherein the cyclic peptide has a minimum inhibitory concentration at which substantially no target microbial organisms grow *in vitro* that is less than one fifth the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells *in vitro*.

4. The pharmaceutical composition of claim 1, wherein the cyclic peptide has a minimum inhibitory concentration at which substantially no target microbial organisms grow *in vitro* that is less than one tenth the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells *in vitro*.

5. The pharmaceutical composition of claim 1, wherein the cyclic peptide has a minimum inhibitory concentration at which substantially no target microbial organisms grow *in vitro* that is less than one twentieth the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells *in vitro*.

6.   The pharmaceutical composition of claim 1, wherein the cyclic peptide causes substantially no hemolysis of mammalian red blood cells *in vitro*.

5   7.   The pharmaceutical composition of claim 1, wherein the target microbial organism is a gram-positive bacterium or a gram-negative bacterium.

8.   The pharmaceutical composition of claim 1, wherein the cyclic peptide self-assembles into a supramolecular structure that is more disruptive of

10   cellular membranes of the target microbial organism than of animal cell membranes.

9.   The pharmaceutical composition of claim 8, wherein the supramolecular structure comprises a nanotube, a barrel of associated, axially parallel

15   nanotubes, a carpet of associated nanotubes, or mixtures thereof.

10.   The pharmaceutical composition of claim 8, wherein the supramolecular structure induces depolarization of the target microbial organism selectively over membrane depolarization of animal cells.

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11.   The pharmaceutical composition of claim 8, wherein the supramolecular structure induces lysis of the target microbial organism selectively over animal cell lysis.

25   12.   The pharmaceutical composition of claim 1, wherein the cyclic peptide comprises a plurality of amino acids having side chains with affinity for biomolecules integral to membranes of the target microbial organism.

13.   The pharmaceutical composition of claim 1, wherein the effective

30   amount is an amount of the cyclic peptide sufficient to induce lysis of target microbial organisms without undesired toxicity against animal cells.

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14.   The pharmaceutical composition of claim 1, wherein the effective
        amount induces substantially no hemolysis of mammalian red blood cells
        in vitro.

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15.   The pharmaceutical composition of claim 1, wherein the effective
        amount is a single dosage.

16.   The pharmaceutical composition of claim 1, wherein the cyclic peptide
10   has at least one polar D- or L-amino acid.

17.   The pharmaceutical composition of claim 1, wherein the cyclic peptide
        has at least two polar D- or L-amino acids.

15   18.   The pharmaceutical composition of claim 1, wherein the cyclic peptide
        has at least three polar D- or L-amino acids.

19.   The pharmaceutical composition of claim 1, wherein the cyclic peptide
        has four to six polar D- or L-amino acids.

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20.   The pharmaceutical composition of any one of claims 17, 18 and 19,
        wherein each polar D- or L-amino acid is adjacent to at least one other
        polar D- or L-amino acid.

25   21.   The pharmaceutical composition of any one of claims 16, 17, 18 and 19,
        wherein at least one polar D- or L-amino acid is adjacent only to nonpolar
        D- or L-amino acids.

22.   The pharmaceutical composition any one of claims 16, 17, 18 and 19,
30   wherein each polar D- or L-amino acid is a D- or L-enantiomer of
        cysteine, homocysteine, serine, threonine, asparagine, glutamine, aspartic

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acid, glutamic acid, histidine, arginine, lysine, hydroxylysine or ornithine.

23.   The pharmaceutical composition of claim 1, wherein the cyclic peptide has at least one ionizable D- or L-amino acid.

24.   The pharmaceutical composition of claim 1, wherein the cyclic peptide has at least two ionizable D- or L-amino acids.

25.   The pharmaceutical composition of claim 1, wherein the cyclic peptide has at least three ionizable D- or L-amino acids.

26.   The pharmaceutical composition of claim 1, wherein the cyclic peptide has four to six ionizable D- or L-amino acids.

27.   The pharmaceutical composition of any one of claims 24, 25 or 26, wherein each ionizable D- or L-amino acid is adjacent to at least one other polar D- or L-amino acid.

28.   The pharmaceutical composition of any one of claims 23, 24, 25 or 26, wherein at least one ionizable D- or L-amino acid is adjacent only to nonpolar D- or L-amino acids.

29.   The pharmaceutical composition of any one of claims 23, 24, 25 or 26, wherein each ionizable D- or L-amino acid is arginine, aspartic acid, glutamic acid, histidine, lysine, hydroxylysine or ornithine.

30.   The pharmaceutical composition of claim 1, wherein the cyclic peptide has at least one nonpolar D- or L-amino acid residue.

31.   The pharmaceutical composition of claim 1, wherein the cyclic peptide has two to fifteen nonpolar D- or L-amino acid residues.

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32. The pharmaceutical composition of claim 30 or 31, wherein each nonpolar D- or L-amino acid residue is alanine, valine, isoleucine, leucine, methionine, norleucine, phenylalanine, tyrosine or tryptophan.
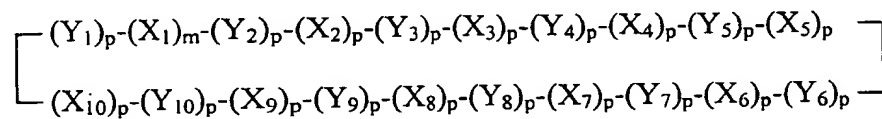
33. A pharmaceutical composition for treating or preventing a microbial infection in an animal comprising a pharmaceutically acceptable carrier and a cyclic peptide having a sequence of about six alternating D- and L-α-amino acids in an amount effective to treat or prevent said infection caused by a target microbial organism in said animal.

34. A pharmaceutical composition for treating or preventing a microbial infection in an animal comprising a pharmaceutically acceptable carrier and a cyclic peptide having a sequence of about eight alternating D- and L-α-amino acids in an amount effective to treat or prevent said infection caused by a target microbial organism in said animal.

35. The pharmaceutical composition of any one of claims 1, 33 or 34, wherein the cyclic peptide has an amino acid sequence of formula I:

$$\left[\begin{array}{l} (Y_1)_p\text{-}(X_1)_m\text{-}(Y_2)_p\text{-}(X_2)_p\text{-}(Y_3)_p\text{-}(X_3)_p\text{-}(Y_4)_p\text{-}(X_4)_p\text{-}(Y_5)_p\text{-}(X_5)_p \\ (X_{10})_p\text{-}(Y_{10})_p\text{-}(X_9)_p\text{-}(Y_9)_p\text{-}(X_8)_p\text{-}(Y_8)_p\text{-}(X_7)_p\text{-}(Y_7)_p\text{-}(X_6)_p\text{-}(Y_6)_p \end{array} \right]$$

I

wherein:

m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a polar D- or L-α-amino acid; and
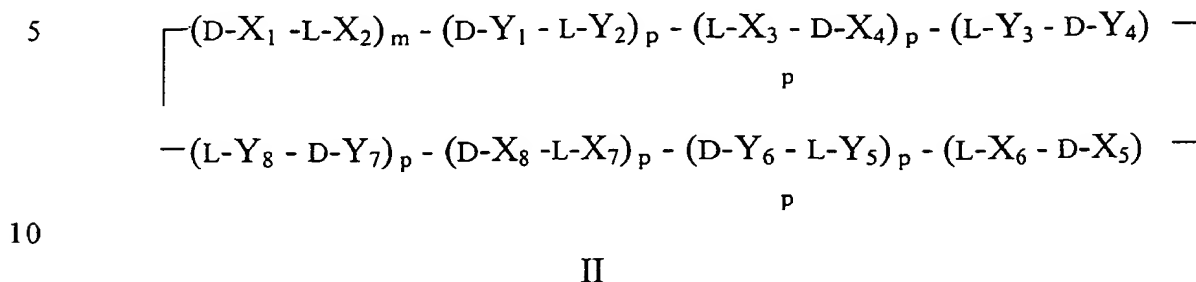
each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately nonpolar D- or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about sixteen alternating D- and L-α amino acids.

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36. The pharmaceutical composition of claim 1, 33 or 34, wherein the cyclic peptide has an amino acid sequence of formula II:

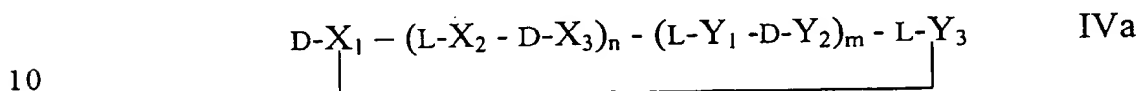$$\left[(D\text{-}X_1\text{-}L\text{-}X_2)_m - (D\text{-}Y_1 - L\text{-}Y_2)_p - (L\text{-}X_3 - D\text{-}X_4)_p - (L\text{-}Y_3 - D\text{-}Y_4)_p \right.$$

$$\left. - (L\text{-}Y_8 - D\text{-}Y_7)_p - (D\text{-}X_8\text{-}L\text{-}X_7)_p - (D\text{-}Y_6 - L\text{-}Y_5)_p - (L\text{-}X_6 - D\text{-}X_5)_p \right.$$

II

wherein:

m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, and X_8 is separately a polar D- or L-α-amino acid;

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, and Y_8 is separately nonpolar D- or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about sixteen alternating D- and L-α amino acids.

37. The pharmaceutical composition any one of claims 1, 33 or 34, wherein the cyclic peptide has an amino acid sequence having having formula III:

$$\left[(X_1)_p\text{-}(X_2)_p\text{-}(X_3)_p\text{-}(X_4)_m\text{-}(X_5)_p\text{-}(X_6)_p\text{-}(X_7)_p\text{-}(X_8)_p\text{-}(X_9)_p\text{-}(X_{10})_p \right.$$

$$\left. (Y_{10})_p\text{-}(Y_9)_p\text{-}(Y_8)_p\text{-}(Y_7)_p\text{-}(Y_6)_p\text{-}(Y_5)_p\text{-}(Y_4)_p\text{-}(Y_3)_p\text{-}(Y_2)_p\text{-}(Y_1)_p \right]$$

III
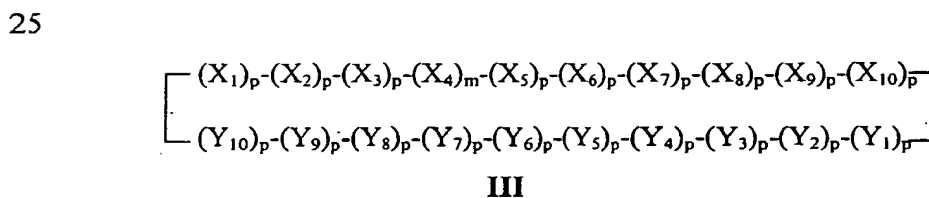
wherein:

m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a polar D- or L-α-amino acid;

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each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately
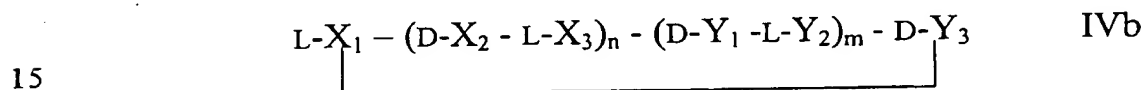
nonpolar D- or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

5

38.   The pharmaceutical composition any one of claims 1, 33 or 34, wherein

the cyclic peptide has an amino acid sequence of formula IVa or IVb:

$$\text{D-X}_1 - (\text{L-X}_2 - \text{D-X}_3)_n - (\text{L-Y}_1 - \text{D-Y}_2)_m - \text{L-Y}_3 \qquad \text{IVa}$$

10

or

$$\text{L-X}_1 - (\text{D-X}_2 - \text{L-X}_3)_n - (\text{D-Y}_1 - \text{L-Y}_2)_m - \text{D-Y}_3 \qquad \text{IVb}$$

15

wherein:

n is an integer ranging from 0 to 4;

m is an integer ranging from 1 to 7;

20   X_1, X_2 and X_3 are each a separate a polar amino acid;

Y_1, Y_2 and Y_3 are each a separate nonpolar amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

25   39.   The pharmaceutical composition of claim 1, 33 or 34, wherein the cyclic

peptide has an amino acid sequence of formula Va or Va:

$$\text{D-X}_1 - \text{L-X}_2 - (\text{D-Y}_1 - \text{L-Y}_2)_q \qquad \text{or} \qquad \text{L-X}_1 - \text{D-X}_2 - (\text{L-Y}_1 - \text{D-Y}_2)_q$$

30

Va   **Vb**
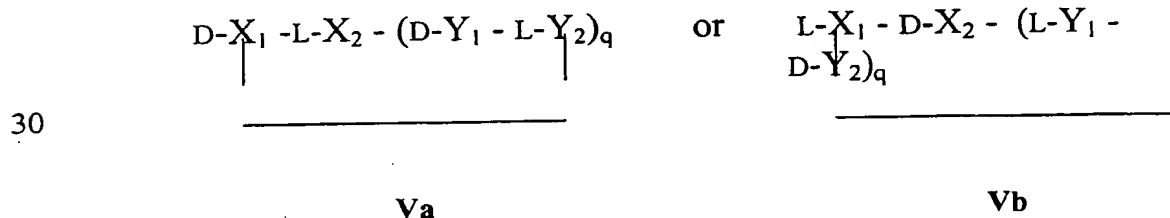
wherein:

q is an integer ranging from 2 to 7;

35   X_1 and X_2 are separately polar amino acids;

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Y$_1$ and Y$_2$ are separately nonpolar amino acids.

40.   The composition of claim 1, wherein the composition comprises a mixture of two or more different cyclic peptides.

41.   The pharmaceutical composition of claim 1, wherein the amino acid sequence comprises any one of SEQ ID NO:8, 9, 12, 17, 18, 26, 29, 47-52, 61, 63, 67, 68, 72-77, 84, 85, 87-89, 91-93, 100, 102, 107, 111, 112, 119, 125 or 139.

42.   A pharmaceutical composition for treating or preventing a microbial infection in an animal comprising a pharmaceutically acceptable carrier and a cyclic peptide having a homochiral sequence of from three to about ten β-amino acids, wherein at least one β-amino acid has at least one polar side chain, in an amount effective to treat or prevent said infection caused by a target microbial organism in said animal.

43.   The pharmaceutical composition of claim 42, wherein the cyclic peptide has a minimum inhibitory concentration at which substantially no target microbial organisms grow *in vitro* that is from about less than one twentieth to less than one half of the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells *in vitro*.

44.   The pharmaceutical composition of claim 42, wherein the cyclic peptide causes substantially no hemolysis of mammalian red blood cells *in vitro*.

45.   The pharmaceutical composition of claim 42, wherein the target microbial organism is a gram-positive bacterium or a gram-negative bacterium.

46.   The pharmaceutical composition of claim 42, wherein the cyclic peptide self-assembles into a supramolecular structure more disruptive of the

membranes of the target microbial organism than of animal cell
membranes.

47. The pharmaceutical composition of claim 46, wherein the supramolecular
     structure comprises a nanotube, a barrel of associated, axially parallel
     nanotubes, a carpet of associated nanotubes, or mixtures thereof.

48. The pharmaceutical composition of claim 46, wherein the supramolecular
     structure induces depolarization of membranes of the target microbial
     organisms selectively over animal cell membrane depolarization.

49. The pharmaceutical composition of claim 46, wherein the supramolecular
     structure induces lysis of target microbial organisms selectively over
     animal cell lysis.

50. The pharmaceutical composition of claim 42, wherein the effective
     amount is an amount of the cyclic peptide sufficient to induce lysis of
     target microbial organisms without inducing an undesired amount of
     lysis of animal cells.

51. The pharmaceutical composition of claim 42, wherein the effective
     amount induces substantially no hemolysis of red blood cells *in vitro*.

52. The pharmaceutical composition of claim 42, wherein the cyclic peptide
     has at least one polar side chain and at least one non-polar side chain on
     adjacent β amino acids.

53. The pharmaceutical composition of claim 42, wherein the cyclic peptide
     has at least two polar side chains.

54. The pharmaceutical composition of claim 42, wherein the cyclic peptide
     has at least three polar side chains.

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55. The pharmaceutical composition of claim 42, wherein the cyclic peptide has four to five polar side chains.

5   56. The pharmaceutical composition of claim 42, wherein the cyclic peptide has at least one ionizable side chain.

57. The pharmaceutical composition of claim 42, wherein the cyclic peptide has at least two ionizable side chains.

10

58. The pharmaceutical composition of claim 42, wherein the cyclic peptide has at least three ionizable side chains.

59. The pharmaceutical composition of claim 42, wherein the cyclic peptide

15   has four to five ionizable side chains.

60. The pharmaceutical composition of claim 42, wherein the cyclic peptide has at least one nonpolar side chain.

20   61. The pharmaceutical composition of claim 42, wherein the cyclic peptide has two to nine nonpolar side chains.

62. The pharmaceutical composition of claim 42, wherein the cyclic β-peptide has an amino acid sequence of formula IV:

25

$$\begin{bmatrix} -(Z_1)_q-(Z_2)_q-(Z_1)_q-(Z_2)_q-(Z_1)_q-(Z_2)_q-(Z_1)_q-(Z_2)_q-(Z_1)_q-(Z_2)_q- \\ -(Z_2)_q-(Z_1)_q-(Z_2)_q-(Z_1)_q-(Z_2)_q-(Z_1)_q-(Z_2)_q-(Z_1)_q-(Z_2)_q-(Z_1)_q- \end{bmatrix}$$
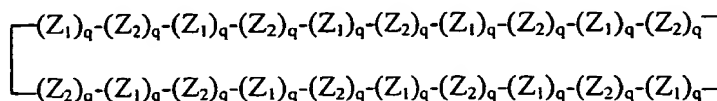
IV

30

wherein:

each q is separately an integer ranging from 0 to 7;

each Z_1, Z_3, Z_5, Z_7, Z_9, Z_{11}, Z_{13}, Z_{15}, Z_{17}, and Z_{19} is separately a

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monosubstituted β-amino acid;

each Z_2, Z_4, Z_6, Z_8, Z_{10}, Z_{12}, Z_{14}, Z_{16}, Z_{18}, and Z_{20} is separately a disubstituted β-amino acid; and

wherein the cyclic β-peptide has a sequence of from three to about ten homochiral β-amino acids, and wherein at least one β-amino acid has at least one polar side chain.

63.   A method of treating a microbial infection in an animal comprising administering to said animal a cyclic peptide comprising a sequence of from four to about sixteen amino acids, wherein the sequence has alternating D- and L-α-amino acids, in an amount sufficient to induce target microbial cell death without inducing an undesirable amount of animal cell death.

64.   The method of claim 63, wherein the cyclic peptide has a minimum inhibitory concentration at which substantially no target microbial organisms grow *in vitro* that is less than one twentieth to less than one half of the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells *in vitro*.

65.   The method of claim 63, wherein the cyclic peptide causes substantially no hemolysis of mammalian red blood cells *in vitro*.

66.   The method of claim 63, wherein the target microbial organism is a gram-positive bacterium or a gram-negative bacterium.

67.   The method of claim 63, wherein the cyclic peptide self-assembles into a supramolecular structure more disruptive of cellular membranes of the target microbial organism than of animal cell membranes.

68. The method of claim 67, wherein the supramolecular structure comprises a nanotube, a barrel of associated, axially parallel nanotubes, a carpet of associated nanotubes, or mixtures thereof.

5 69. The method of claim 67, wherein the supramolecular structure induces depolarization of membranes of the target microbial organism selectively over animal cell membrane depolarization.

70. The method of claim 63, wherein the supramolecular structure induces

10 lysis of the target microbial organism selectively over lysis of animal cells.

71. The method of claim 63, wherein the amount sufficient to induce target microbial cell death is an amount of the cyclic peptide sufficient to

15 induce lysis of microbial cells without inducing an undesired amount of lysis of animal cells.

72. The method of claim 63, wherein the amount sufficient to induce target microbial cell death induces substantially no hemolysis of mammalian

20 red blood cells *in vitro*.

73. The method of claim 63, wherein the cyclic peptide has at least one polar D- or L-amino acid.

25 74. The method of claim 63, wherein the cyclic peptide has at least two polar D- or L-amino acids.

75. The method of claim 63, wherein the cyclic peptide has at least three polar D- or L-amino acids.

30

76. The method of claim 63, wherein the cyclic peptide has four to six polar D- or L-amino acids.

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77. The method of any one of claims 74, 75 and 76, wherein each polar D- or L-amino acid is adjacent to at least one other polar D- or L-amino acid.

5 78. The method of any one of claims 74, 75 and 76, wherein at least one polar D- or L-amino acid is adjacent only to nonpolar D- or L-amino acids.

79. The method of any one of claims 73, 74, 75 and 76, wherein each polar D- or L-amino acid is separately a D- or L-enantiomer of cysteine,

10 homocysteine, serine, threonine, asparagine, glutamine, aspartic acid, glutamic acid, histidine, arginine, lysine, hydroxylysine or ornithine.

80. The method of claim 63, wherein the cyclic peptide has at least one ionizable D- or L-amino acid.

15

81. The method of claim 63, wherein the cyclic peptide has at least two ionizable D- or L-amino acids.

82. The method of claim 63, wherein the cyclic peptide has at least three

20 ionizable D- or L-amino acids.

83. The method of claim 63, wherein the cyclic peptide has four to six ionizable D- or L-amino acids.

25 84. The method of any one of claims 81, 82 or 83, wherein each ionizable D- or L-amino acid is adjacent to at least one other polar D- or L-amino acid.

85. The method of any one of claims 81, 82 or 83, wherein at least one ionizable D- or L-amino acid is adjacent only to nonpolar D- or L-amino

30 acids.

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86.   The method of any one of claims 80, 81, 82 or 83, wherein each ionizable D- or L-amino acid is separately a D- or L-enantiomer of arginine, aspartic acid, glutamic acid, histidine, lysine, hydroxylysine or ornithine.

5   87.   The method of claim 63, wherein the cyclic peptide has at least one nonpolar D- or L-amino acid residue.

88.   The method of claim 63, wherein the cyclic peptide has two to fifteen nonpolar D- or L-amino acid residues.

10

89.   The method of claim 87 or 88, wherein each nonpolar D- or L-amino acid residue is a D- or L-alanine, valine, isoleucine, leucine, methionine, norleucine, phenylalanine, tyrosine or tryptophan.

15   90.   A method of treating an infection caused by a target microbial organism in an animal comprising administering to said animal a cyclic peptide comprising a sequence of about six amino acids, wherein the sequence has alternating D- and L-α-amino acids, in an amount sufficient to induce target microbial cell death without inducing an undesirable amount of

20   animal cell death.

91.   A method of treating an infection caused by a target microbial organism in an animal, comprising administering to said animal a cyclic peptide comprising a sequence of about eight amino acids, wherein the sequence

25   has alternating D- and L-α-amino acids, in an amount sufficient to induce target microbial cell death without inducing an undesirable amount of animal cell death.

92.   The method of any one of claims 63, 90 or 91, wherein the cyclic peptide

30   has an amino acid sequence of formula I:

$$\lceil (Y_1)_p\text{-}(X_1)_m\text{-}(Y_2)_p\text{-}(X_2)_p\text{-}(Y_3)_p\text{-}(X_3)_p\text{-}(Y_4)_p\text{-}(X_4)_p\text{-}(Y_5)_p\text{-}(X_5)_p \rceil$$

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$$- (X_{10})_p\text{-}(Y_{10})_p\text{-}(X_9)_p\text{-}(Y_9)_p\text{-}(X_8)_p\text{-}(Y_8)_p\text{-}(X_7)_p\text{-}(Y_7)_p\text{-}(X_6)_p\text{-}(Y_6)_p -$$
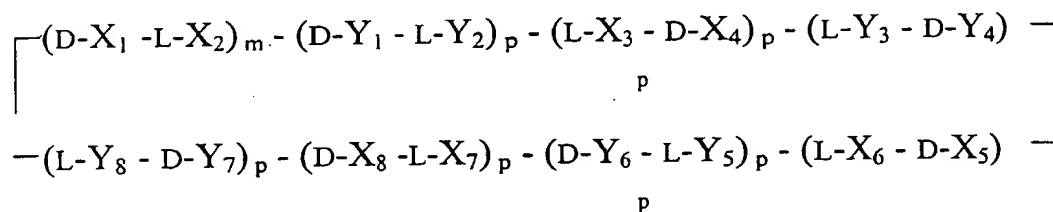
I
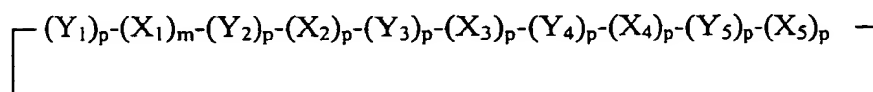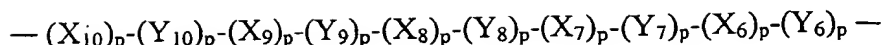
wherein:

m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a polar D- or L-α-amino acid; and

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately nonpolar D- or L-α-amino acid; and

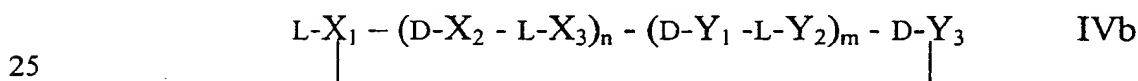wherein the cyclic peptide has an even number of from four to about sixteen alternating D- and L-α amino acids.

93.   The method of any one of claims 63, 90 or 91, wherein the cyclic peptide has an amino acid sequence of formula II:

$$\left\lceil (D\text{-}X_1\text{-}L\text{-}X_2)_m - (D\text{-}Y_1\text{-}L\text{-}Y_2)_p - (L\text{-}X_3\text{-}D\text{-}X_4)_p - (L\text{-}Y_3\text{-}D\text{-}Y_4)_p \right.$$

$$\left. -(L\text{-}Y_8\text{-}D\text{-}Y_7)_p - (D\text{-}X_8\text{-}L\text{-}X_7)_p - (D\text{-}Y_6\text{-}L\text{-}Y_5)_p - (L\text{-}X_6\text{-}D\text{-}X_5)_p \right\rceil$$

II

wherein:

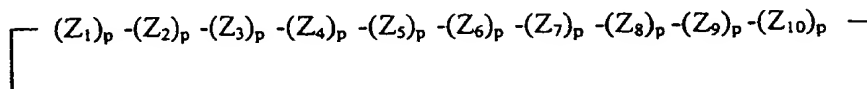m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, and X_8 is separately a polar D- or L-α-amino acid;

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, and Y_8 is separately nonpolar D- or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about sixteen alternating D- and L-α amino acids.

94.   The method of any one of claims 63, 90 or 91, wherein the cyclic peptide has an amino acid sequence having having formula III:

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$$\left[\begin{array}{l} (X_1)_p\text{-}(X_2)_p\text{-}(X_3)_p\text{-}(X_4)_m\text{-}(X_5)_p\text{-}(X_6)_p\text{-}(X_7)_p\text{-}(X_8)_p\text{-}(X_9)_p\text{-}(X_{10})_p \\ (Y_{10})_p\text{-}(Y_9)_p\text{-}(Y_8)_p\text{-}(Y_7)_p\text{-}(Y_6)_p\text{-}(Y_5)_p\text{-}(Y_4)_p\text{-}(Y_3)_p\text{-}(Y_2)_p\text{-}(Y_1)_p \end{array} \right]$$

III

wherein:

m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a

polar D- or L-α-amino acid;

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately

nonpolar D- or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

95.   The method of any one of claims 63, 90 or 91, wherein the cyclic peptide

has an amino acid sequence of formula IVa or IVb:

$$\text{D-}X_1 - (\text{L-}X_2 - \text{D-}X_3)_n - (\text{L-}Y_1 - \text{D-}Y_2)_m - \text{L-}Y_3 \qquad \text{IVa}$$

or

$$\text{L-}X_1 - (\text{D-}X_2 - \text{L-}X_3)_n - (\text{D-}Y_1 - \text{L-}Y_2)_m - \text{D-}Y_3 \qquad \text{IVb}$$

wherein:

n is an integer ranging from 0 to 4;

m is an integer ranging from 1 to 7;

X_1, X_2 and X_3 are each a separate a polar amino acid;

Y_1, Y_2 and Y_3 are each a separate nonpolar amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

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96.   The method of any one of claims 63, 90 or 91, wherein the cyclic peptide has an amino acid sequence of formula Va or Va:

$$\text{D-X}_1\text{-L-X}_2 - (\text{D-Y}_1 - \text{L-Y}_2)_q \qquad \text{or} \qquad \text{L-X}_1 - \text{D-X}_2 - (\text{L-Y}_1 - \text{D-Y}_2)_q$$

Va   **Vb**
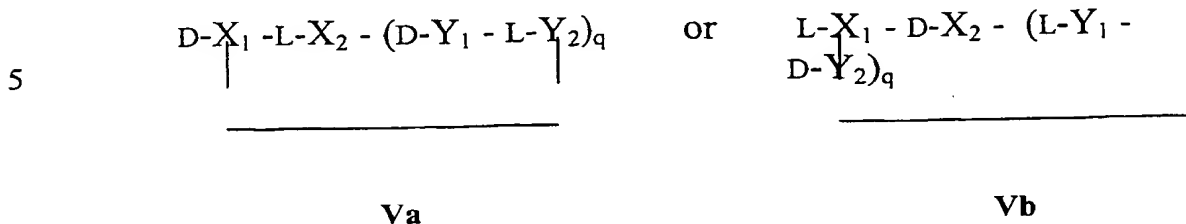
wherein:

q is an integer ranging from 2 to 7;

X_1 and X_2 are separately polar amino acids;

Y_1 and Y_2 are separately nonpolar amino acids.

97.   The method of claim 63, wherein the composition comprises a mixture of two or more different cyclic peptides.

98.   The method of claim 63, wherein the amino acid sequence comprises any one of SEQ ID NO:8, 9, 12, 17, 18, 26, 29, 47-52, 61, 63, 67, 68, 72-77, 84, 85, 87-89, 91-93, 100, 102, 107, 111, 112, 119, 125 or 139.

99.   A method of treating or preventing an infection caused by a target microbial organism in an animal comprising administering to said mammal a cyclic peptide comprising a sequence of from three to about ten β-amino acids, in an amount sufficient to induce death of said target microbial organisms without inducing an undesirable amount of animal cell death.

100.   The method of claim 99, wherein the cyclic peptide has a minimum inhibitory concentration at which substantially no target microbial organisms grow in vitro that is less than one twentieth to less than one half of the peptide concentration needed to cause 50% hemolysis of mammalian red blood cells *in vitro*.

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101. The method of claim 99, wherein the cyclic peptide causes substantially no hemolysis of mammalian red blood cells *in vitro*.

102. The method of claim 99, wherein the target microbial organism is a gram-positive bacterium or a gram-negative bacterium.

103. The method of claim 99, wherein the cyclic peptide self-assembles into a supramolecular structure more disruptive of cell membranes of target microbial organisms than of animal cell membranes.

104. The method of claim 103, wherein the supramolecular structure comprises a nanotube, a barrel of associated, axially parallel nanotubes, a carpet of associated nanotubes, or mixtures thereof.

105. The method of claim 103, wherein the supramolecular structure induces depolarization of membranes of target microbial organisms selectively over animal cell membrane depolarization.

106. The method of claim 103, wherein the supramolecular structure induces lysis of target microbial organisms selectively over animal cell lysis.

107. The method of claim 99, wherein the amount sufficient to induce death of target microbial organisms is an amount of the cyclic peptide sufficient to induce lysis of microbial cells without inducing an undesired amount of lysis of animal cells.

108. The method of claim 99, wherein the amount sufficient to induce death of target microbial organisms induces substantially no hemolysis of red blood cells *in vitro*.

109. The method of claim 99, wherein the cyclic peptide has at least one polar side chain.

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110. The method of claim 99, wherein the cyclic peptide has at least two polar side chains.

5 111. The method of claim 99, wherein the cyclic peptide has at least three side chains.

112. The method of claim 99, wherein the cyclic peptide has four to five polar side chains.

10

113. The method of claim 99, wherein the cyclic peptide has at least one ionizable side chain.

114. The method of claim 99, wherein the cyclic peptide has at least two
15 ionizable side chains.

115. The method of claim 99, wherein the cyclic peptide has at least three ionizable side chains.

20 116. The method of claim 99, wherein the cyclic peptide has four to five ionizable side chains.

117. The method of claim 99, wherein the cyclic peptide has at least one nonpolar side chain.

25

118. The method of claim 99, wherein the cyclic peptide has two to nine nonpolar side chains.

119. The method of claim 99, wherein the cyclic β-peptide can have an amino
30 acid sequence of formula VI:
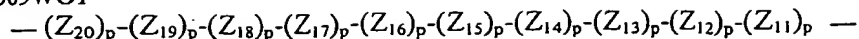
$$\left[(Z_1)_p\ \text{-}(Z_2)_p\ \text{-}(Z_3)_p\ \text{-}(Z_4)_p\ \text{-}(Z_5)_p\ \text{-}(Z_6)_p\ \text{-}(Z_7)_p\ \text{-}(Z_8)_p\ \text{-}(Z_9)_p\ \text{-}(Z_{10})_p \right]$$

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$$— (Z_{20})_p\text{-}(Z_{19})_p\text{-}(Z_{18})_p\text{-}(Z_{17})_p\text{-}(Z_{16})_p\text{-}(Z_{15})_p\text{-}(Z_{14})_p\text{-}(Z_{13})_p\text{-}(Z_{12})_p\text{-}(Z_{11})_p —$$

VI

wherein:

each p is separately an integer ranging from 0 to 7;

each Z_1, Z_3, Z_5, Z_7, Z_9, Z_{11}, Z_{13}, Z_{15}, Z_{17}, and Z_{19} is separately a monosubstituted β-amino acid;

each Z_2, Z_4, Z_6, Z_8, Z_{10}, Z_{12}, Z_{14}, Z_{16}, Z_{18}, and Z_{20} is separately a disubstituted β-amino acid; and

wherein the cyclic β-peptide has a sequence of from three to about ten homochiral β-amino acid side chains.

120. A method for identifying or evaluating a cyclic peptide with anti-microbial activity comprising:

(a) contacting a target microbial organism with a test cyclic peptide; and

(b) determining whether the test cyclic peptide has anti-microbial activity;

wherein the test cyclic peptide comprises (i) a sequence of from four to about sixteen alternating D- and L- α-amino acids, or (ii) a sequence of from three to about ten β-amino acids.

121. A method for identifying or evaluating a cyclic peptide selectively cytotoxic to a target microbial organism comprising:

(a) obtaining a combinatorial library of test cyclic peptides, wherein each test cyclic peptide in the combinatorial library comprises (i) an alternating D- and L- α amino acid sequence of from four to about sixteen amino acids, or (ii) a sequence of from three to about ten β-amino acids;

(b) contacting a target microbial cell with one or more of the test cyclic peptides; and

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(c) determining whether one or more test cyclic peptides have anti-microbial activity.

122.   A method of identifying a cyclic peptide selectively cytotoxic to a target microbial organism, comprising:

    (a) identifying a first cyclic peptide from the combinatorial library of claim 121 that can induce cell death of said target microbial organism;

    (b) exchanging at least one amino acid for a different amino acid in the sequence of the first cyclic peptide to generate a second cyclic peptide;

    (c) contacting a target microbial cell with the second cyclic peptide; and

    (d) determining whether the second cyclic peptide has anti-microbial activity.

123.   The method of any one of claims 120, 121 or 122, wherein the method further comprises determining whether a cyclic peptide induces an undesired amount of lysis of an animal cell type.

124.   The method of any one of claims 123, wherein the animal cell type is a mammalian red blood cell.

125.   The method of any one of claims 120, 121 or 122, wherein the microbe is a bacterium, a yeast strain, a fungus, or a single cell parasite.

126.   The method of any one of claims 123, wherein the method further comprises determining whether a cyclic peptide induces substantial cell death in another animal cell type.

127.   The method of any one of claims 120, 121 or 122, wherein the contacting step is performed *in vitro*.

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128.   The method of any one of claims 120, 121 or 122, wherein the contacting step is performed *in vivo*.

5   129.   The method of identifying a cyclic peptide capable of selective association with a target biomolecule, comprising contacting a solution of cyclic peptides with the target biomolecule and determining whether the peptides assemble into a supramolecular structure that selectively associates with the biomolecule, wherein the cyclic peptides comprise an

10   alternating D- and L-α amino acid sequence of from four to about sixteen amino acids.

130.   The method of claim 129, wherein the target biomolecule is displayed on the surface of a living cell.

15

131.   The method of claim 129, wherein the target biomolecule is displayed on the surface of a liposome.

132.   The method of claim 129, wherein more than one peptide is contacted

20   with the target biomolecule.

133.   The method of claim 129, wherein the supramolecular structure comprises a nanotube, a barrel of associated, axially parallel nanotubes, a carpet of associated nanotubes, or mixtures thereof.

25

134.   A peptide comprising a cyclic amino acid sequence of from four to about sixteen alternating D- and L-α-amino acids, said peptide having anti-microbial activity but substantially no undesired anti-animal cell activity.

30   135.   The peptide of claim 134, wherein the peptide has a minimum inhibitory concentration at which substantially no target microbial organisms grow *in vitro* that is less than about one twentieth to about one half of the

peptide concentration needed to cause 50% hemolysis of mammalian red blood cells.

136. The peptide of claim 134, wherein the peptide causes substantially no hemolysis of mammalian red blood cells *in vitro*.

137. The peptide of claim 134, wherein the anti-microbial activity is against gram-positive bacteria or gram-negative bacteria.

138. The peptide of claim 134, wherein the cyclic peptide self-assembles into a supramolecular structure more disruptive of target microbial membranes than of animal cell membranes.

139. The peptide of claim 134, wherein the supramolecular structure comprises a nanotube, a barrel of associated, axially parallel nanotubes, a carpet of associated nanotubes, or mixtures thereof.

140. The peptide of claim 134, wherein the supramolecular structure induces microbial cell membrane depolarization selectively over animal cell membrane depolarization.

141. The peptide of claim 134, wherein the supramolecular structure induces microbial cell lysis selectively over animal cell lysis.

142. The peptide of claim 134, wherein the peptide induces lysis of microbial cells without inducing an undesired amount of lysis of animal cells.

143. The peptide of claim 134, wherein the peptide induces substantially no hemolysis of mammalian red blood cells *in vitro*.

144. The peptide of claim 134, wherein the peptide has at least one polar D- or L-amino acid.

145. The peptide of claim 134, wherein the peptide has at least two polar D- or L-amino acids.

5 146. The peptide of claim 134, wherein the peptide has at least three polar D- or L-amino acids.

147. The peptide of claim 134, wherein the peptide has four to six polar D- or L-amino acids.

10

148. The peptide of any one of claims 145, 146 or 147, wherein each polar D- or L-amino acid is adjacent to at least one other polar D- or L-amino acid.

149. The peptide of any one of claims 145, 146 or 147, wherein at least one
15 polar D- or L-amino acid is adjacent only to nonpolar D- or L-amino acids.

150. The peptide of any one of claims 144, 145, 146 or 147, wherein each polar D- or L-amino acid is separately a D- or L-enantiomer of cysteine, homocysteine, serine, threonine, asparagine, glutamine, aspartic acid,
20 glutamic acid, histidine, arginine, lysine, hydroxylysine or ornithine.

151. The peptide of claim 134, wherein the cyclic peptide has at least one ionizable D- or L-amino acid.

25 152. The peptide of claim 134, wherein the cyclic peptide has at least two ionizable D- or L-amino acids.

153. The peptide of claim 134, wherein the cyclic peptide has at least three ionizable D- or L-amino acids.

30

154. The peptide of claim 134, wherein the cyclic peptide has four to six ionizable D- or L-amino acids.

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155.   The peptide of any one of claims 152, 153 or 154, wherein each ionizable D- or L-amino acid is adjacent to at least one other polar D- or L-amino acid.

5

156.   The peptide of any one of claims 152, 153 or 154, wherein at least one ionizable D- or L-amino acid is adjacent only to nonpolar D- or L-amino acids

10   157.   The peptide of any one of claims 151, 152, 153 or 154, wherein each ionizable D- or L-amino acid is separately arginine, aspartic acid, glutamic acid, histidine, lysine, hydroxylysine or ornithine.

158.   The peptide of claim 134, wherein the cyclic peptide has at least one
15   nonpolar D- or L-amino acid residue.

159.   The peptide of claim 134, wherein the cyclic peptide has two to fifteen nonpolar D- or L-amino acid residues.

20   160.   The peptide of claim 158 or 159, wherein each nonpolar D- or L-amino acid residue is alanine, valine, isoleucine, leucine, methionine, norleucine, phenylalanine, tyrosine or tryptophan.

161.   A peptide comprising a cyclic amino acid sequence of about six
25   alternating D- and L-α-amino acids, said peptide having anti-microbial activity but substantially no anti-animal cell activity.

162.   A peptide comprising a cyclic amino acid sequence of about eight alternating D- and L-α-amino acids, said peptide having anti-microbial
30   activity but substantially no anti-animal cell activity.

163.   The peptide of any one of claims 134, 161 or 162, wherein the cyclic

peptide has an amino acid sequence of formula I:

5

$$\left[\begin{array}{l} (Y_1)_p\text{-}(X_1)_m\text{-}(Y_2)_p\text{-}(X_2)_p\text{-}(Y_3)_p\text{-}(X_3)_p\text{-}(Y_4)_p\text{-}(X_4)_p\text{-}(Y_5)_p\text{-}(X_5)_p \\ (X_{10})_p\text{-}(Y_{10})_p\text{-}(X_9)_p\text{-}(Y_9)_p\text{-}(X_8)_p\text{-}(Y_8)_p\text{-}(X_7)_p\text{-}(Y_7)_p\text{-}(X_6)_p\text{-}(Y_6)_p \end{array} \right]$$

I

wherein:

m is an integer ranging from 1 to 7;

10   each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a

polar D- or L-α-amino acid; and

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately

nonpolar D- or L-α-amino acid;

15   wherein the cyclic peptide has a sequence comprising an even number of

from four to about sixteen alternating D- and L-α amino acids; and

wherein the sequence is not:

cyclo-[-(Gln- D-Ala)$_4$-];

cyclo-[-(Gln- D-Leu)$_4$-];

20   cyclo-[-(Gln- D-Val)$_4$-];

cyclo-[-(Phe- D-Leu)$_4$-];

cyclo-[-(Phe- D-Ala)$_4$-];

cyclo-[-(Gln- D-Ala-Glu- D-Ala)$_2$-];

cyclo-[-(Gln- D-Phe-Glu- D-Phe)$_2$-];

25   cyclo-[-(Gln- D-Ala-Glu- D-Ala)$_3$-];

cyclo-[-(Trp- D-Leu)$_3$-Gln- D-Leu-]; and

cyclo-[-Gln- (D-Leu-Trp)$_4$- D-Leu-].

164.   The peptide of any one of claims 134, 161 or 162, wherein the cyclic

30   peptide has an amino acid sequence of formula II:

$$\left[(D\text{-}X_1\text{-}L\text{-}X_2)_m - (D\text{-}Y_1 - L\text{-}Y_2)_p - (L\text{-}X_3 - D\text{-}X_4)_p - (L\text{-}Y_3 - D\text{-}Y_4) \right]_p$$

133

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$$-(\text{L-Y}_8 - \text{D-Y}_7)_p - (\text{D-X}_8 - \text{L-X}_7)_p - (\text{D-Y}_6 - \text{L-Y}_5)_p - (\text{L-X}_6 - \text{D-X}_5)_p -$$
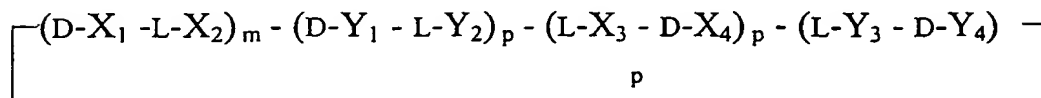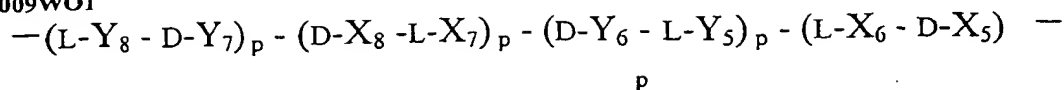
II

5

wherein:

m is an integer ranging from 1 to 7;

each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, and X_8 is separately a polar D- or
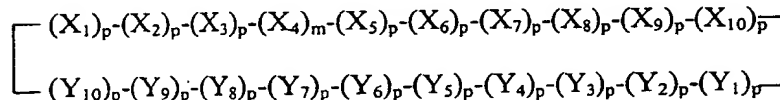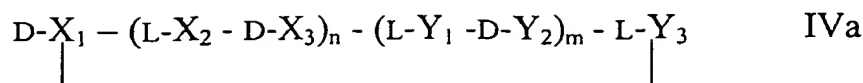
10   L-α-amino acid;

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, and Y_8 is separately nonpolar D-

or L-α-amino acid; and

wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

15

165.   The peptide of any one of claims 134, 161 or 162, wherein the peptide

has an amino acid sequence having having formula III:

20

$$\left[\begin{array}{l} (X_1)_p\text{-}(X_2)_p\text{-}(X_3)_p\text{-}(X_4)_m\text{-}(X_5)_p\text{-}(X_6)_p\text{-}(X_7)_p\text{-}(X_8)_p\text{-}(X_9)_p\text{-}(X_{10})_p \\ (Y_{10})_p\text{-}(Y_9)_p\text{-}(Y_8)_p\text{-}(Y_7)_p\text{-}(Y_6)_p\text{-}(Y_5)_p\text{-}(Y_4)_p\text{-}(Y_3)_p\text{-}(Y_2)_p\text{-}(Y_1)_p \end{array} \right]$$

III

wherein:

each m is separately an integer ranging from 1 to 7;

25   each p is separately an integer ranging from 0 to 7;

each X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, and X_{10} is separately a

polar D- or L-α-amino acid;

each Y_1, Y_2, Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, and Y_{10} is separately

nonpolar D- or L-α-amino acid; and

30   wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

166.   The peptide of any one of claims 134, 161 or 162, wherein the peptide

has an amino acid sequence of formula IVa or IVb:

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$$D\text{-}X_1 - (L\text{-}X_2 - D\text{-}X_3)_n - (L\text{-}Y_1 - D\text{-}Y_2)_m - L\text{-}Y_3 \qquad \text{IVa}$$

5   or

$$L\text{-}X_1 - (D\text{-}X_2 - L\text{-}X_3)_n - (D\text{-}Y_1 - L\text{-}Y_2)_m - D\text{-}Y_3 \qquad \text{IVb}$$
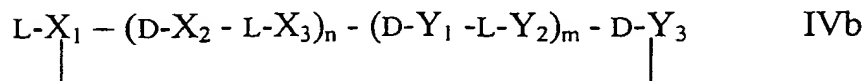
10   wherein:

n is an integer ranging from 0 to 4;

m is an integer ranging from 1 to 7;

X_1, X_2 and X_3 are each a separate a polar amino acid;

Y_1, Y_2 and Y_3 are each a separate nonpolar amino acid; and

15   wherein the cyclic peptide has an even number of from four to about

sixteen alternating D- and L-α amino acids.

167.   The peptide of any one of claims 134, 161 or 162, wherein the peptide

has an amino acid sequence of formula Va or Va:

20

$$D\text{-}X_1 - L\text{-}X_2 - (D\text{-}Y_1 - L\text{-}Y_2)_q \quad \text{or} \quad L\text{-}X_1 - D\text{-}X_2 - (L\text{-}Y_1 - D\text{-}Y_2)_q$$
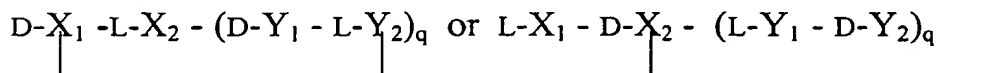
Va   **Vb**

25   wherein:

q is an integer ranging from 2 to 7;

X_1 and X_2 are separately polar amino acids;

Y_1 and Y_2 are separately nonpolar amino acids.

30   168.   The peptide of claim 134, wherein the amino acid sequence comprises

any one of SEQ ID NO:8, 9, 12, 17, 18, 26, 29, 47-52, 61, 63, 67, 68, 72-

77, 84, 85, 87-89, 91-93, 100, 102, 107, 111, 112, 119, 125 or 139.

169.   The pharmaceutical composition of any of claims 1, 33 or 34, wherein

35   the animal is a human.

135

170.   The pharmaceutical composition of any of claims 1, 33 or 34, wherein the animal is a farm animal.

5 171.   The pharmaceutical composition of any of claims 1, 33 or 34, wherein the animal is a domestic animal.

172.   The pharmaceutical composition of claim 42, wherein the animal is a human.

10

173.   The pharmaceutical composition of claim 42, wherein the animal is a farm animal.

174.   The pharmaceutical composition of claim 42, wherein the animal is a
15   domestic animal.

175.   The method of claim 63, wherein the animal is a human.

176.   The method of claim 63, wherein the animal is a farm animal.

20

177.   The method of claim 63, wherein the animal is a domestic animal.

178.   The peptide of any of claims 134, 161 or 162 that is substantially non-toxic in mammals.

25

179.   The peptide of any of claim 134, 161 or 162 that is substantially non-toxic in humans.

180.   The peptide of any of claims 134, 161 or 162 that is substantially non-
30   toxic in a target animal in which the peptide is intended for use to treat or prevent a microbial infection.
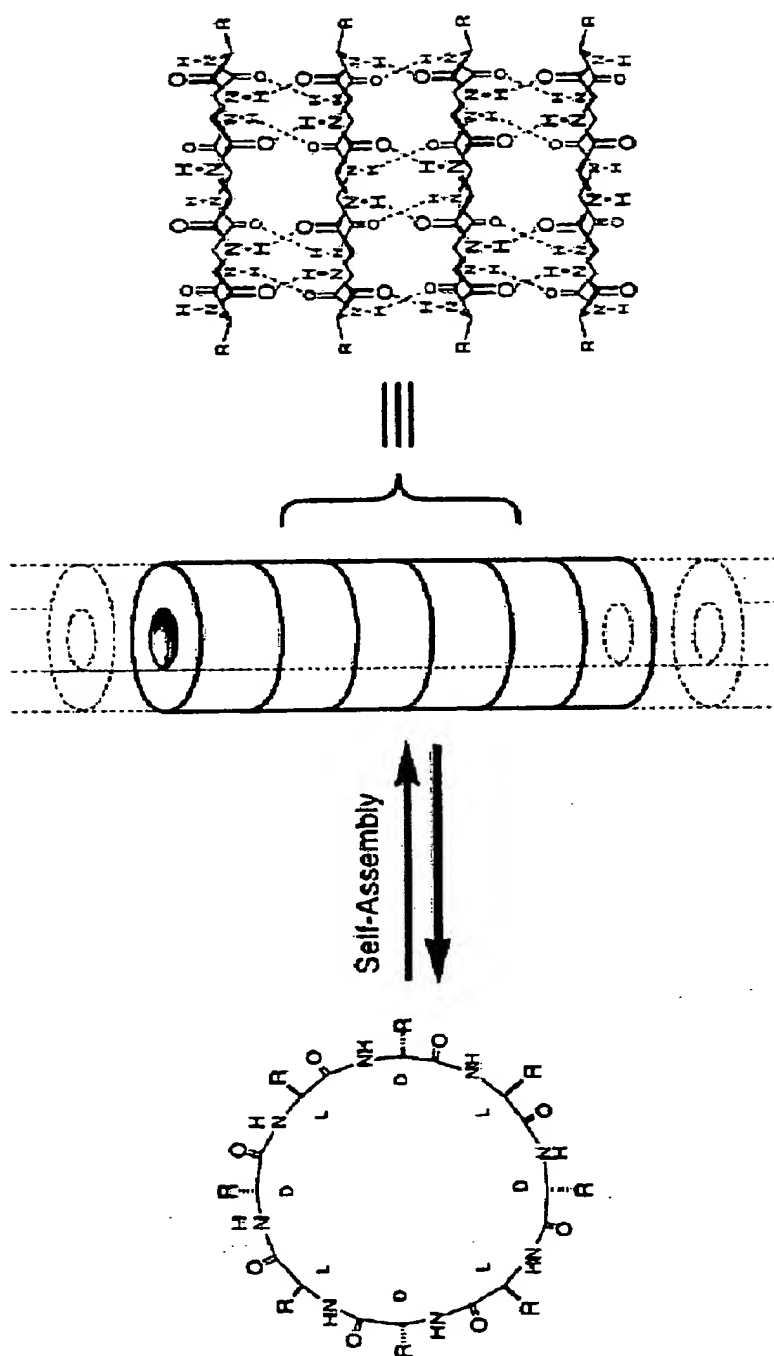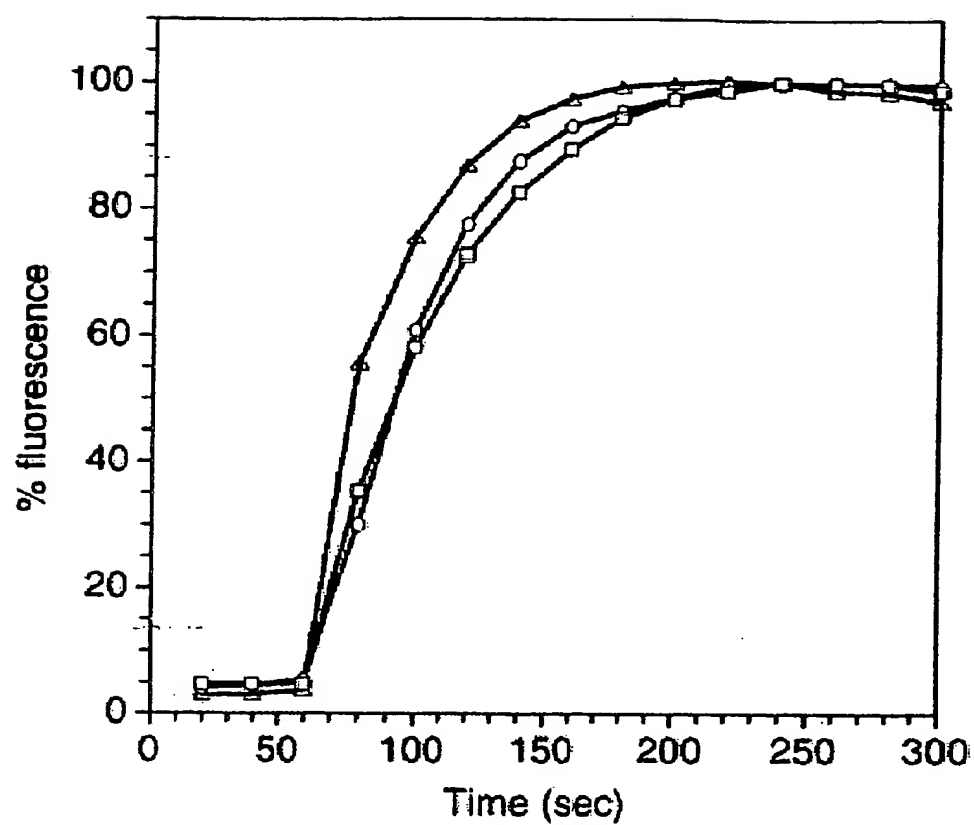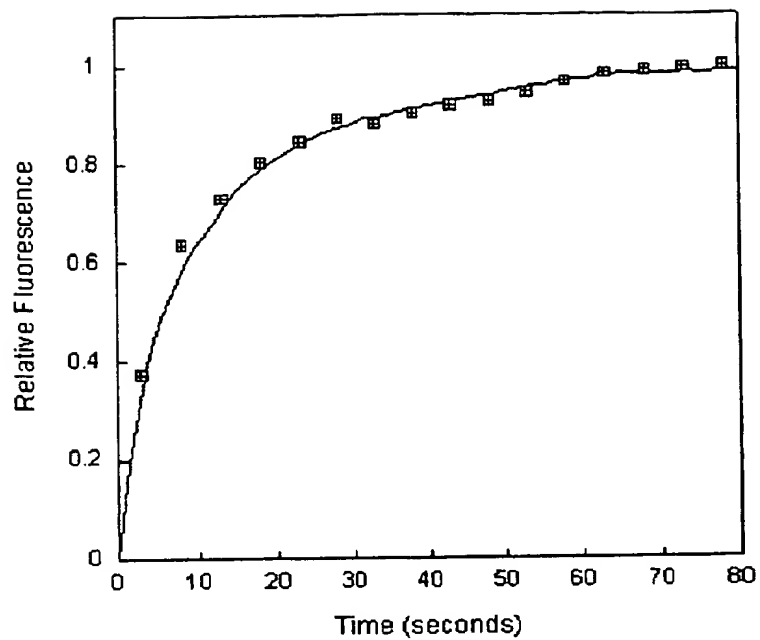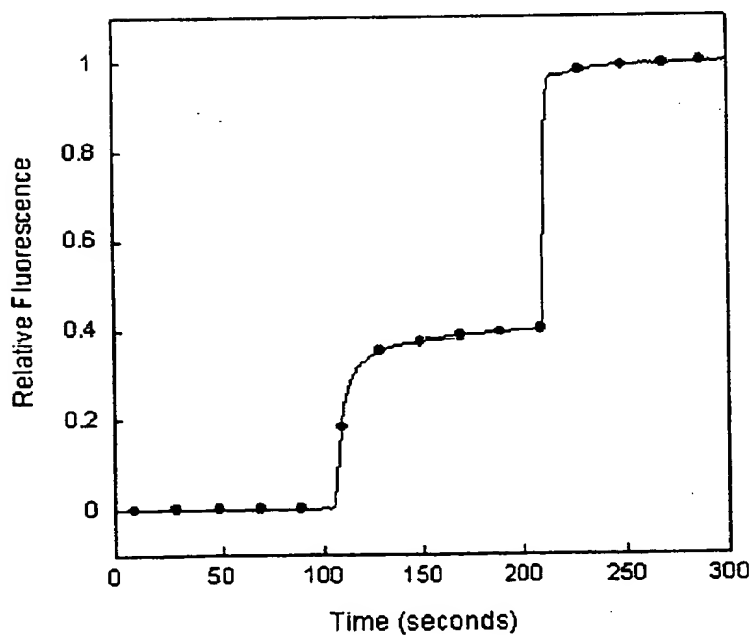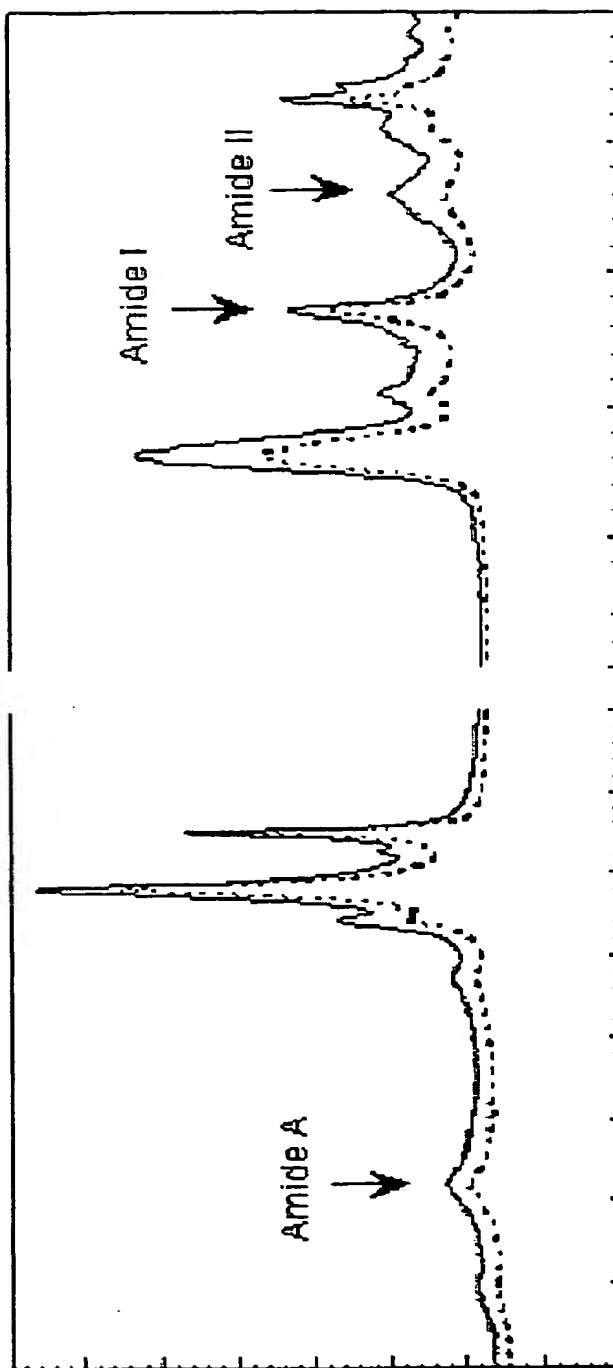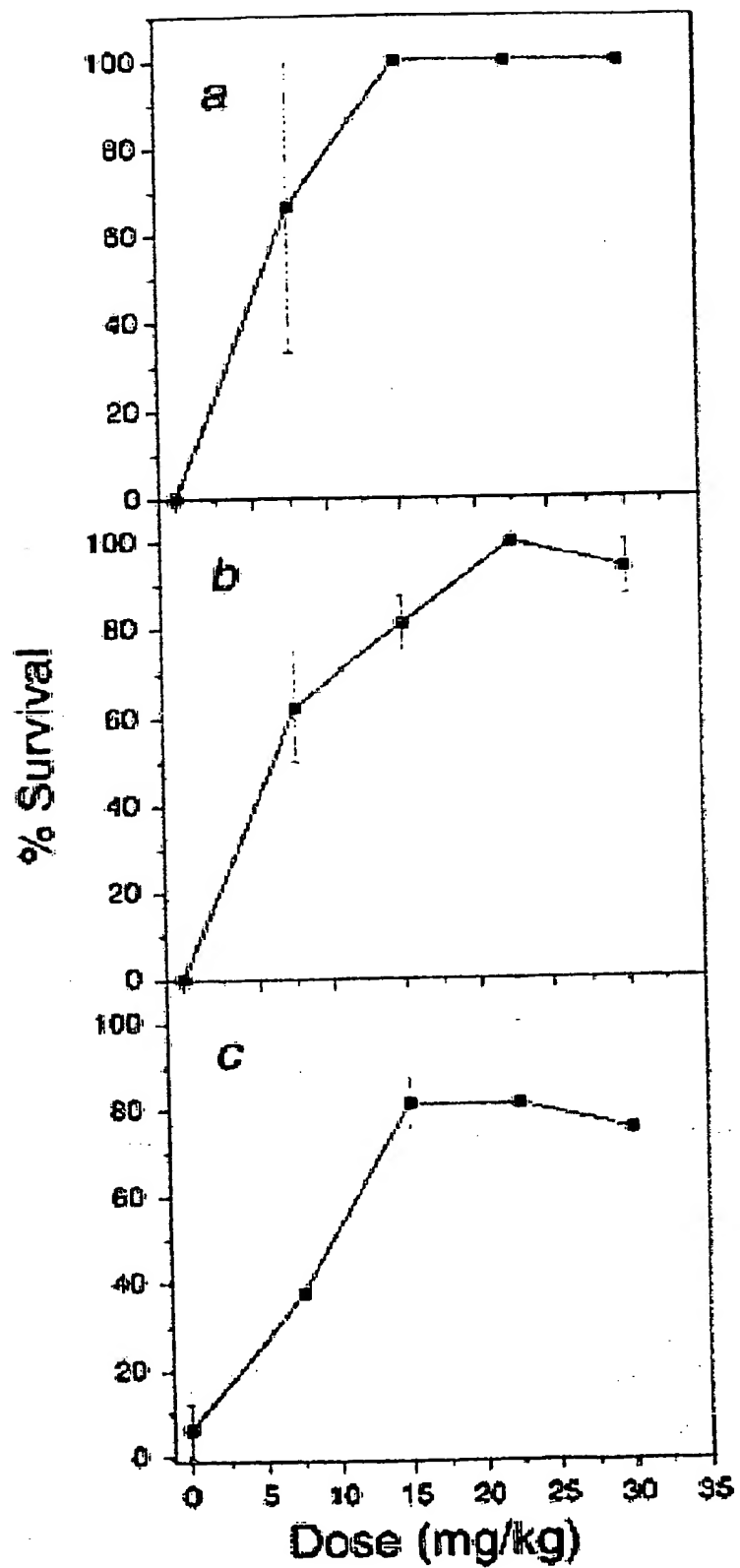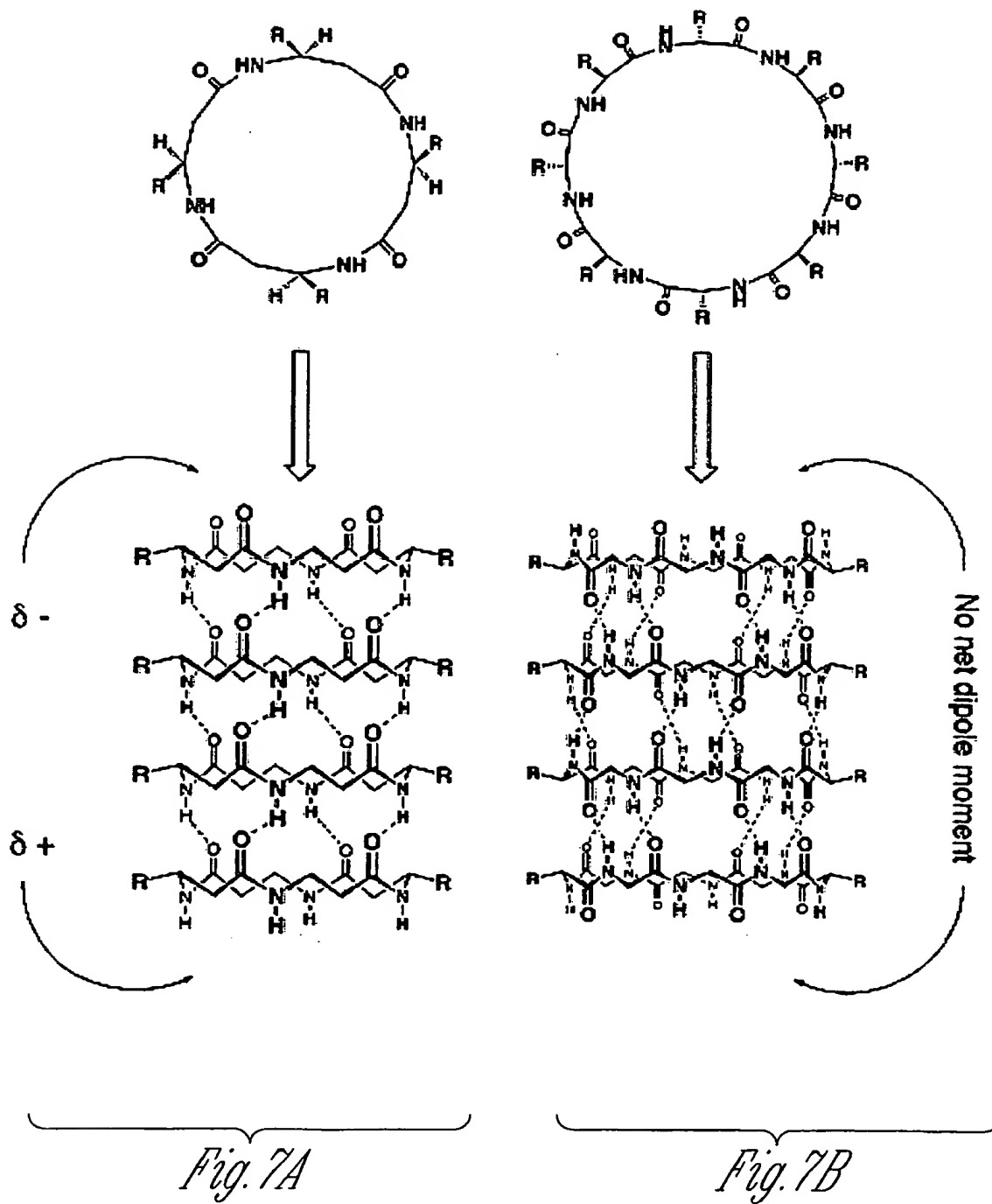
Fig. 1

Fig.2

Fig. 3

Fig. 4A

Fig. 4B

Fig.5

Fig.6

Fig.8B

Fig.8A

Fig. 9
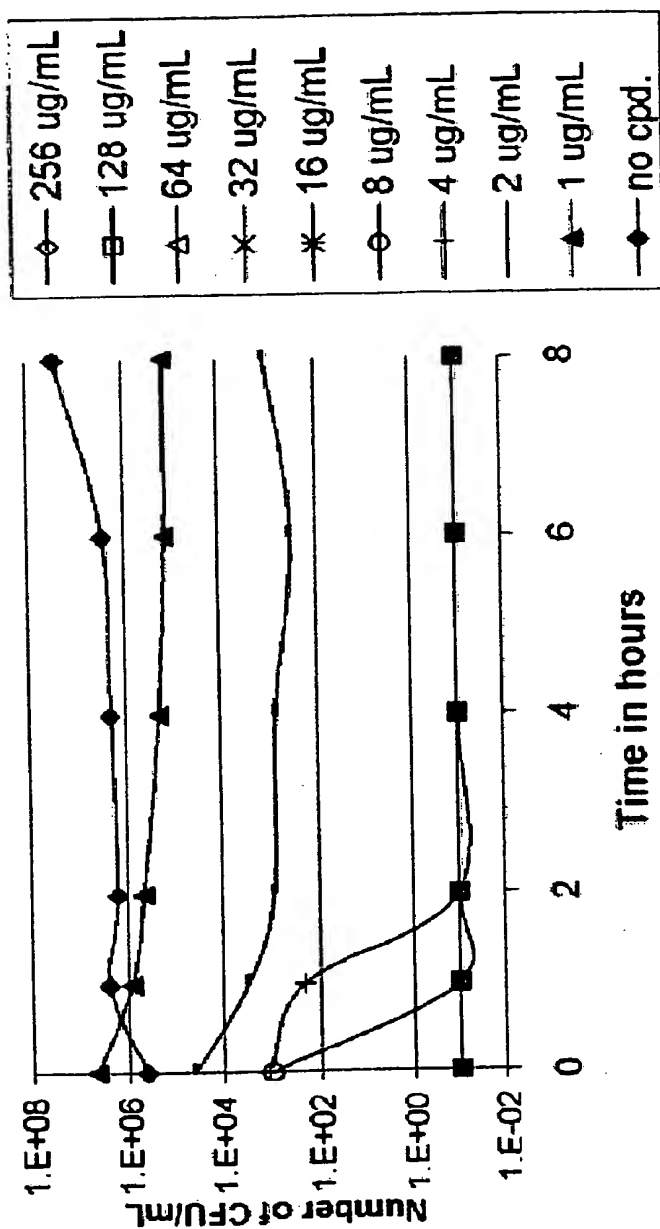
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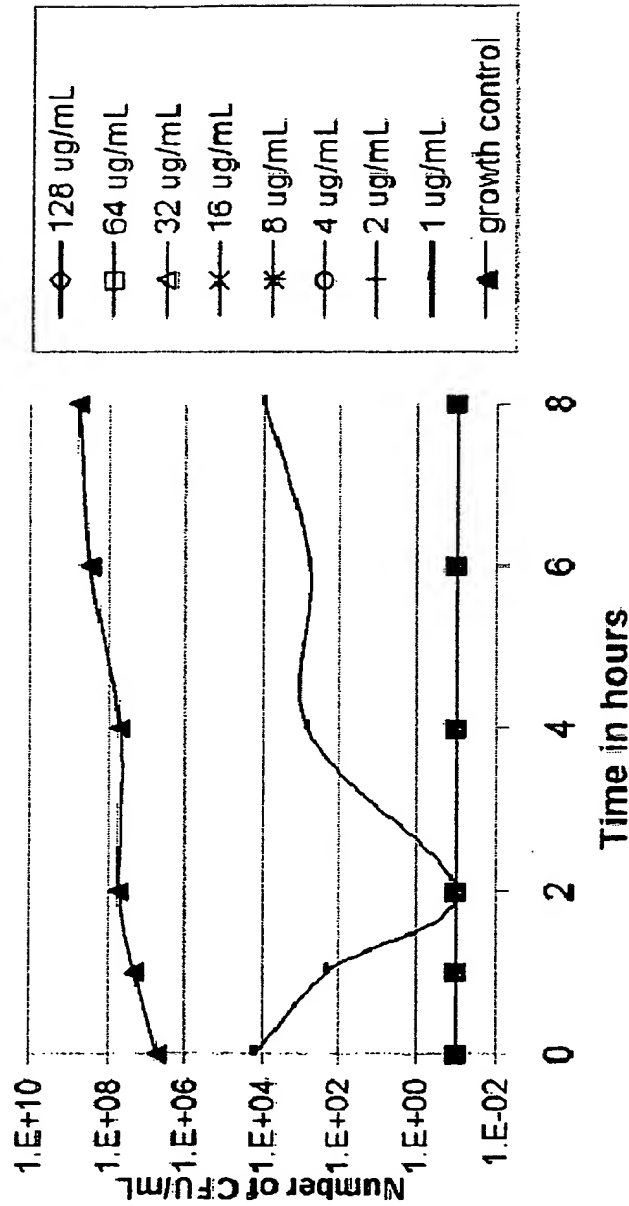
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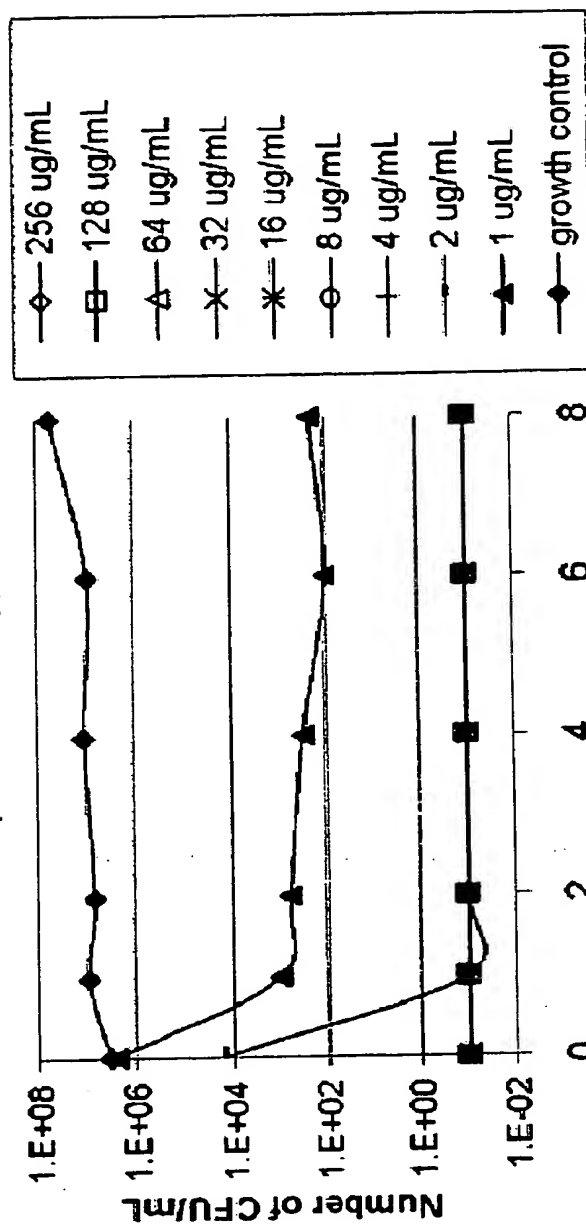
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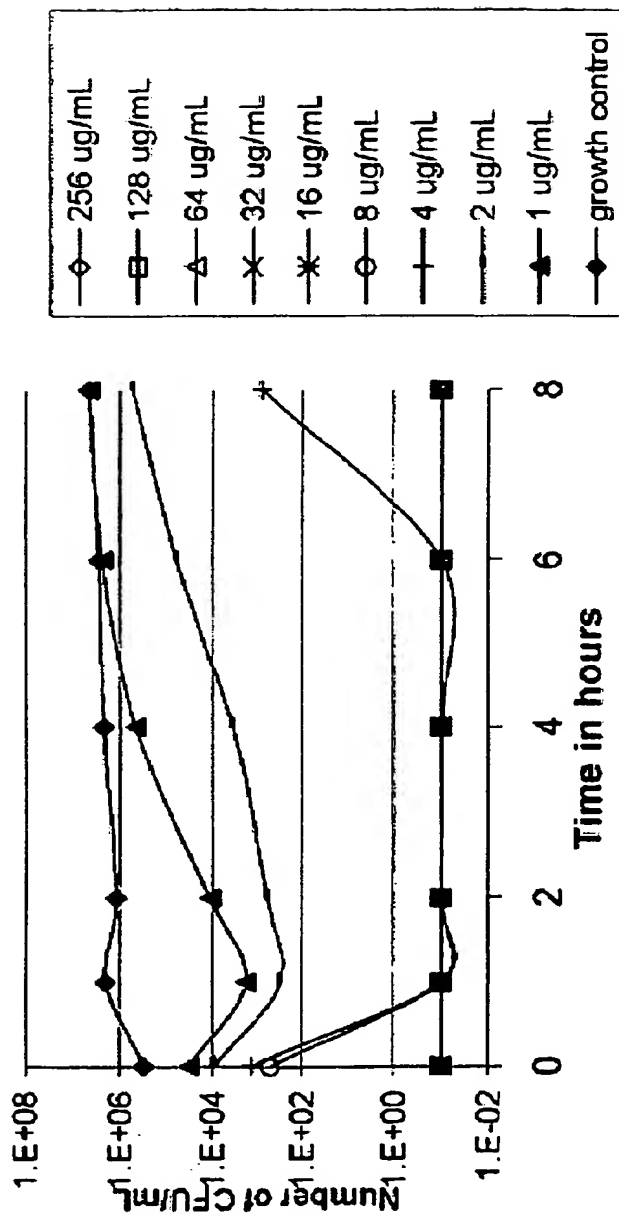
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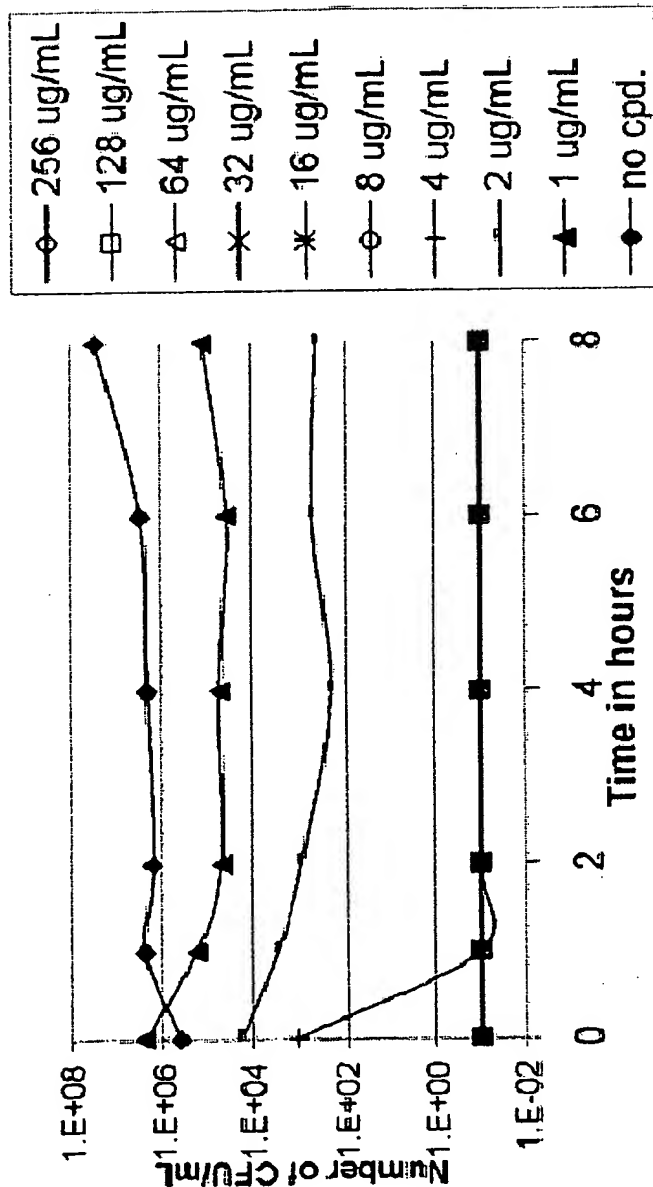
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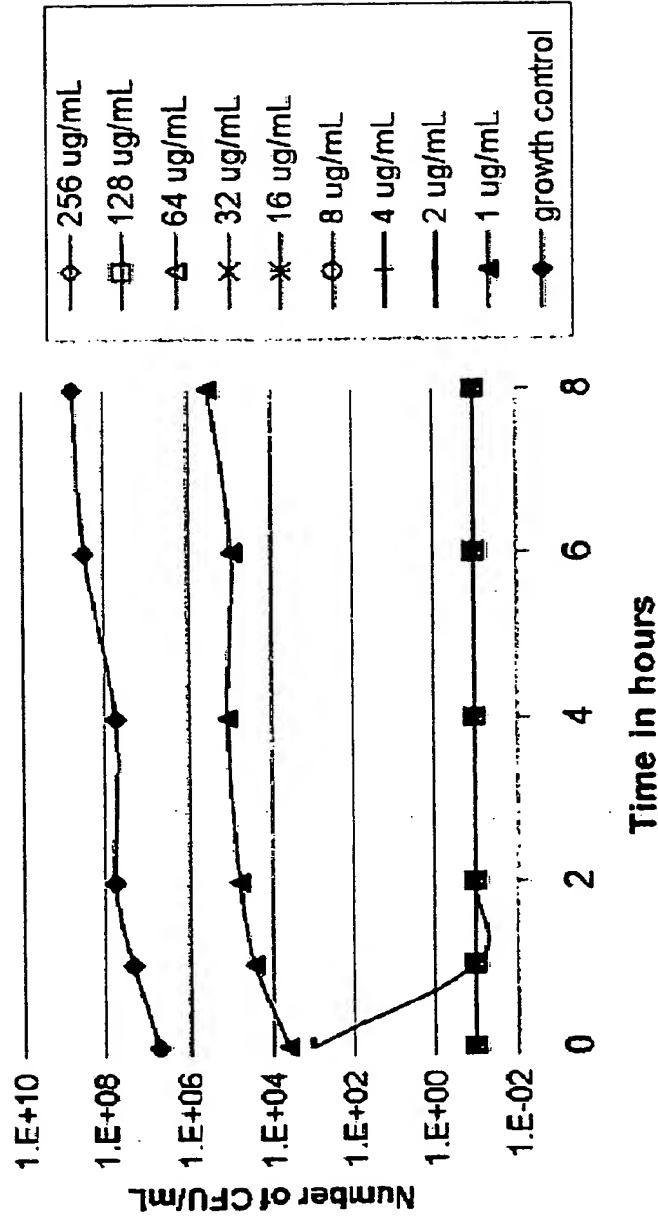
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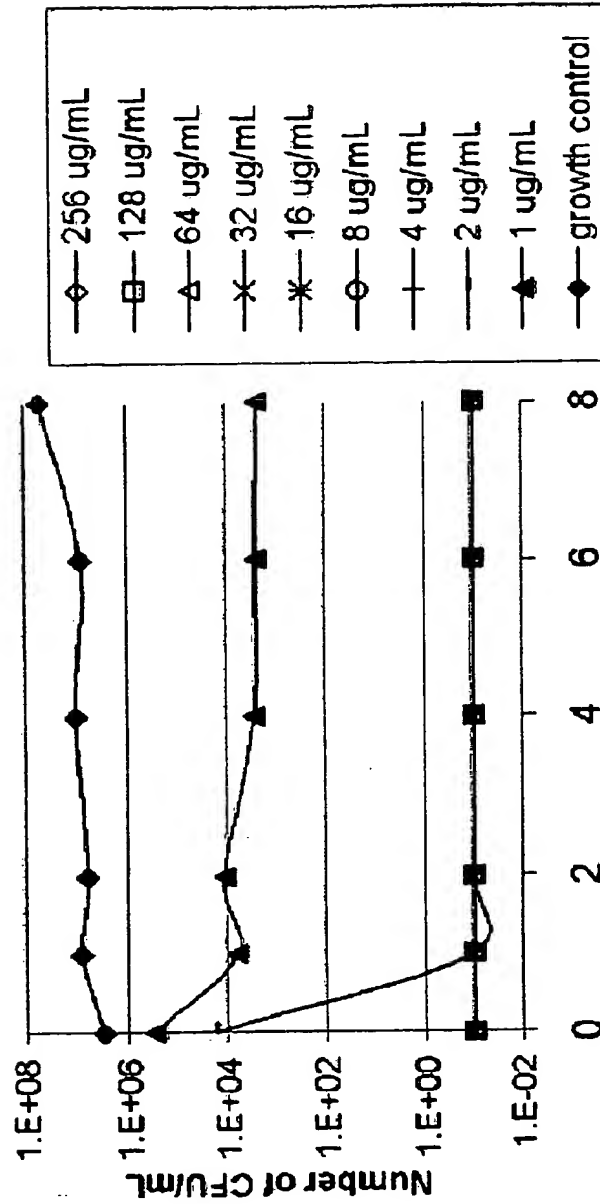
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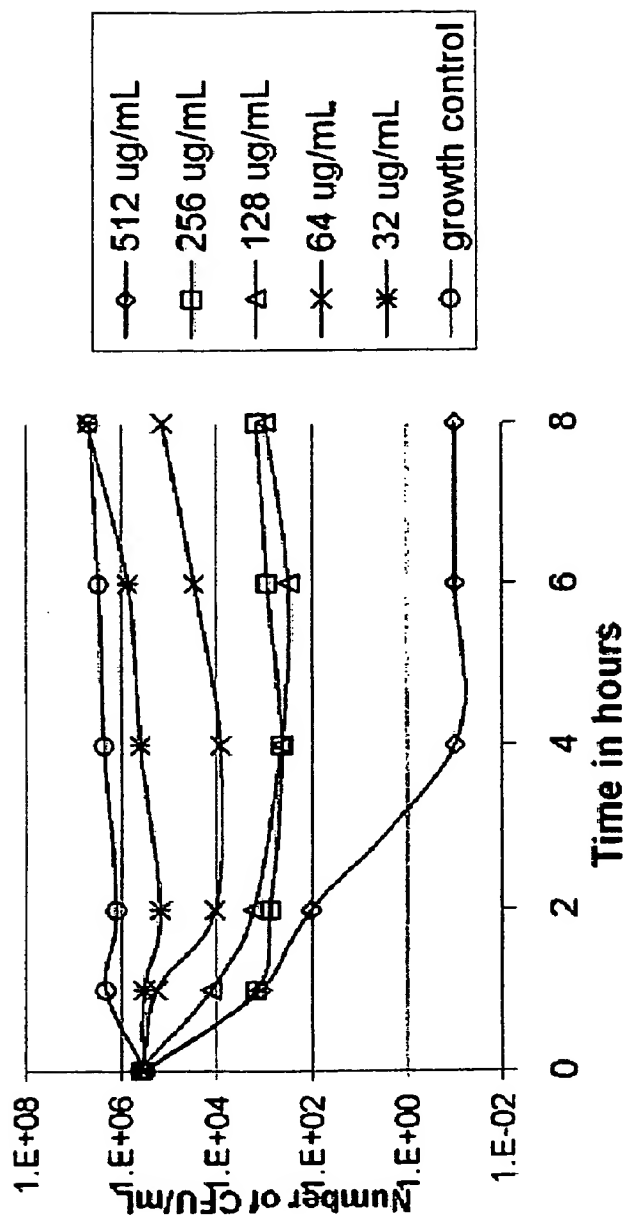
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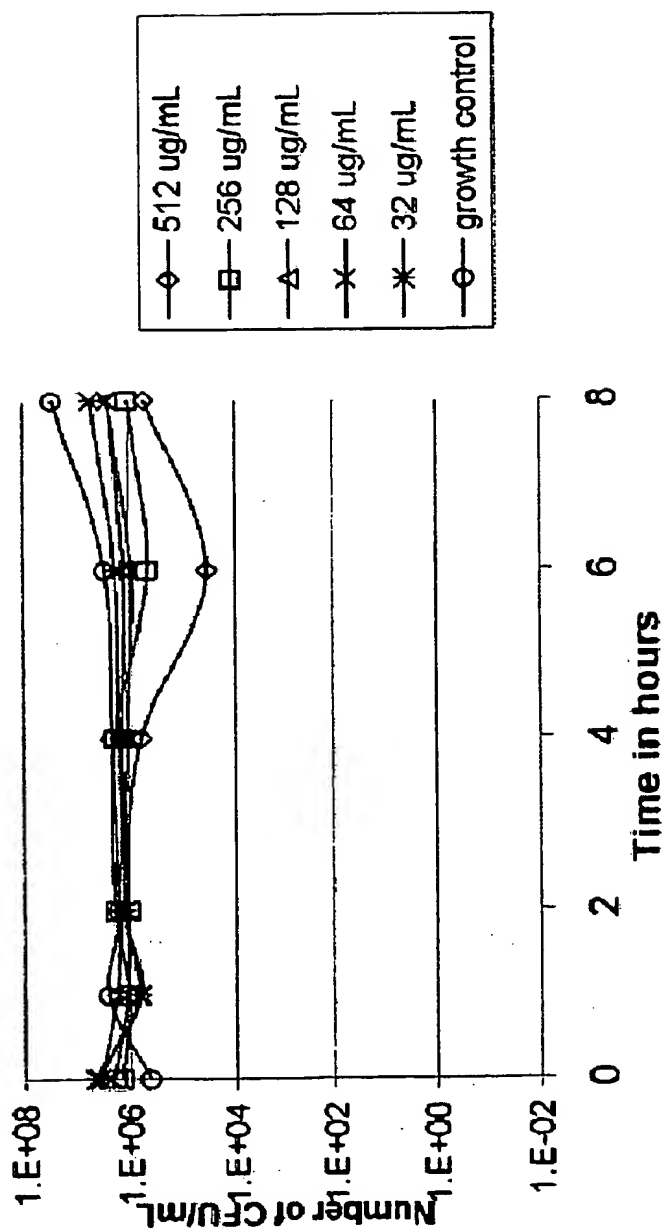
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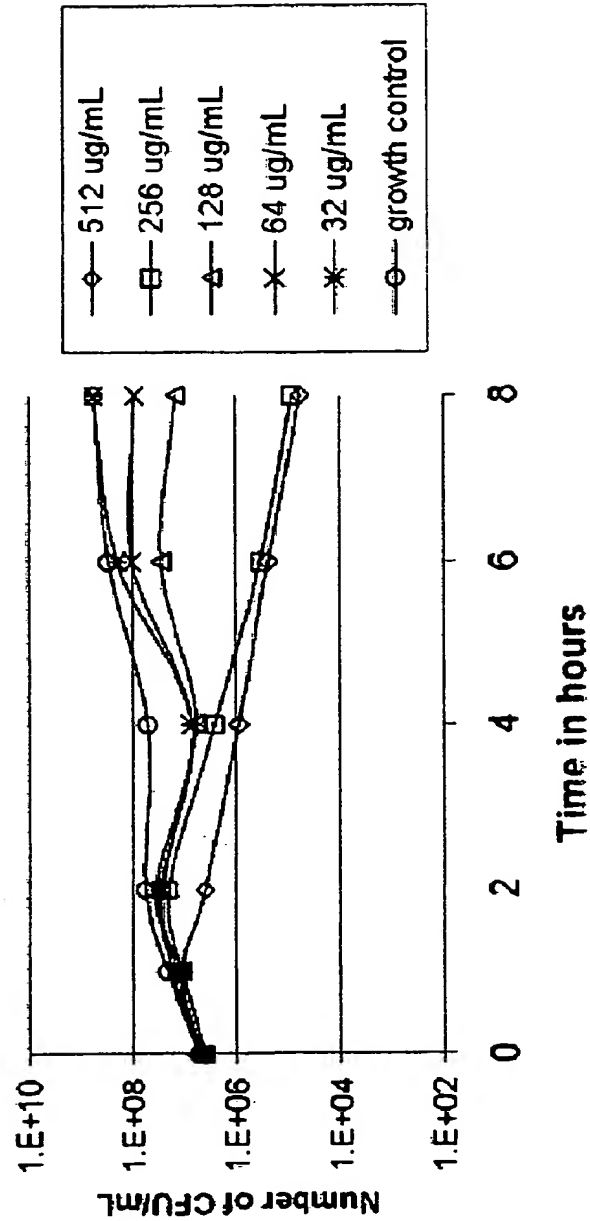
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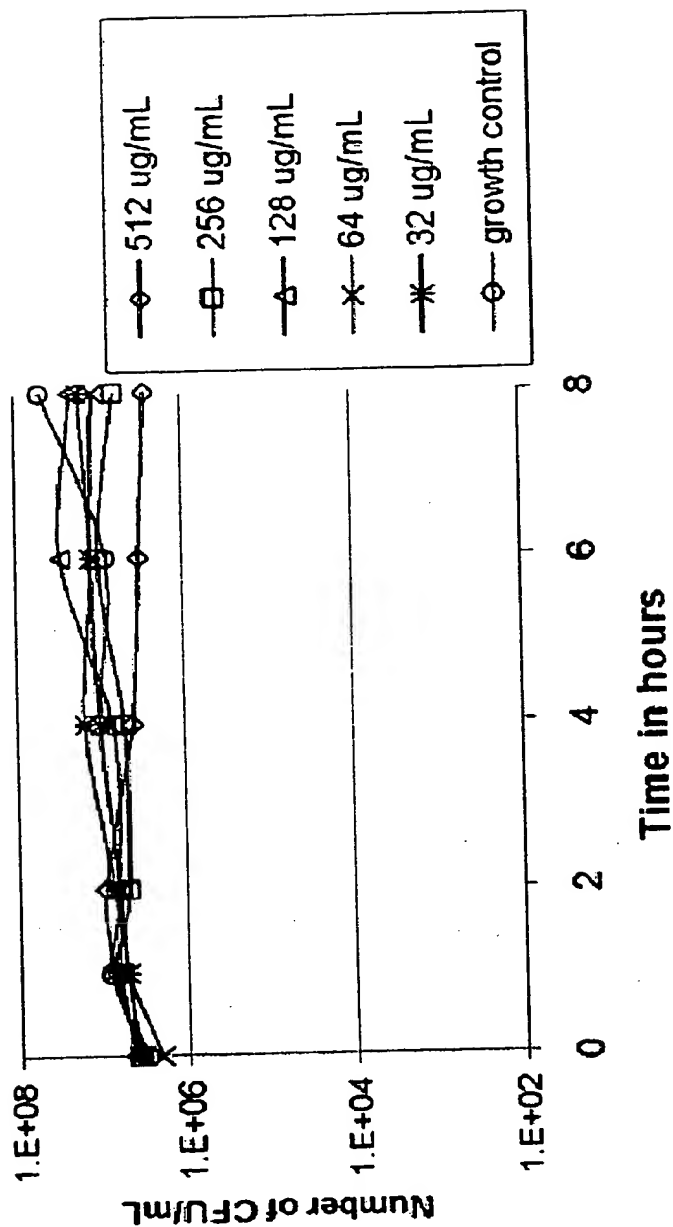
Fig.20

Fig.21

Fig.22
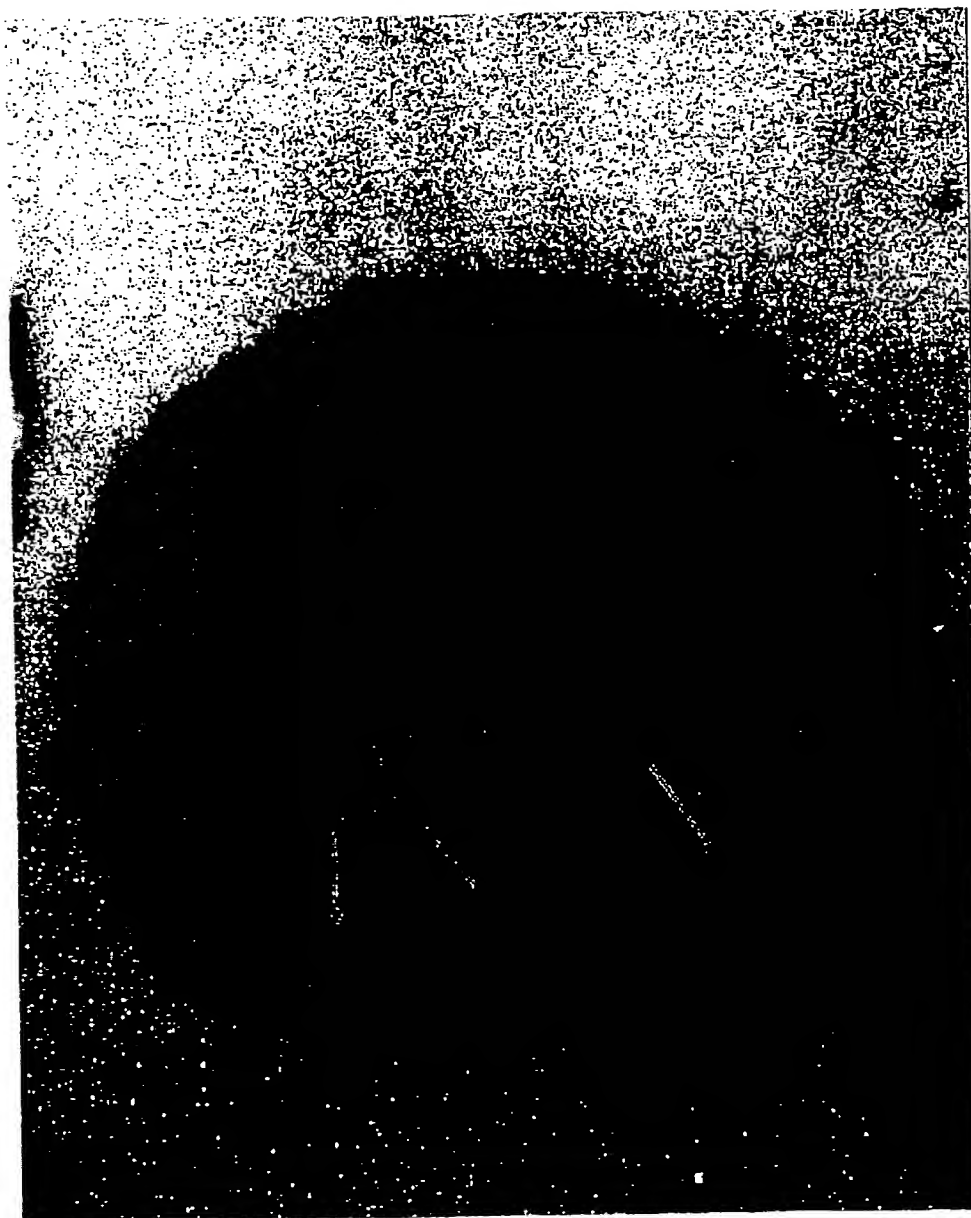
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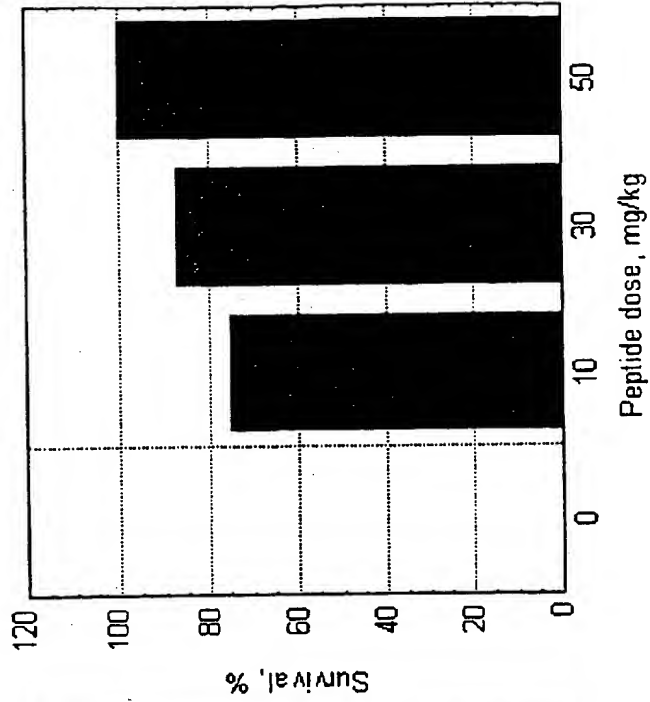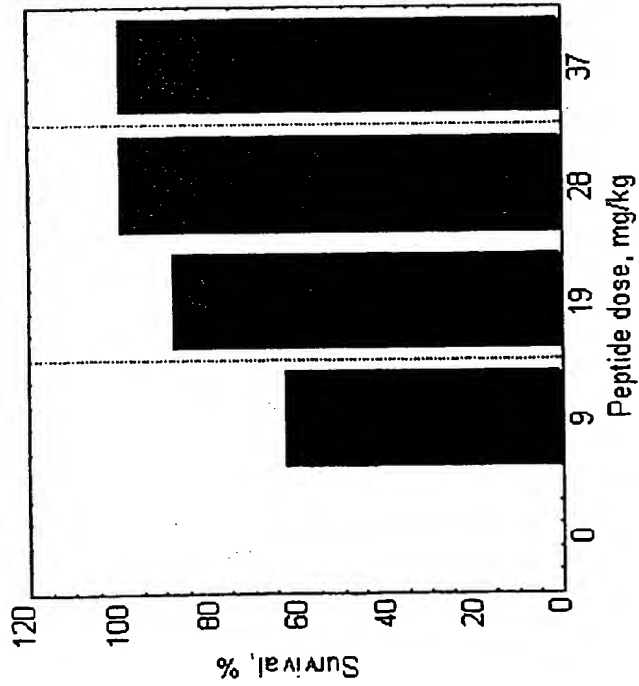
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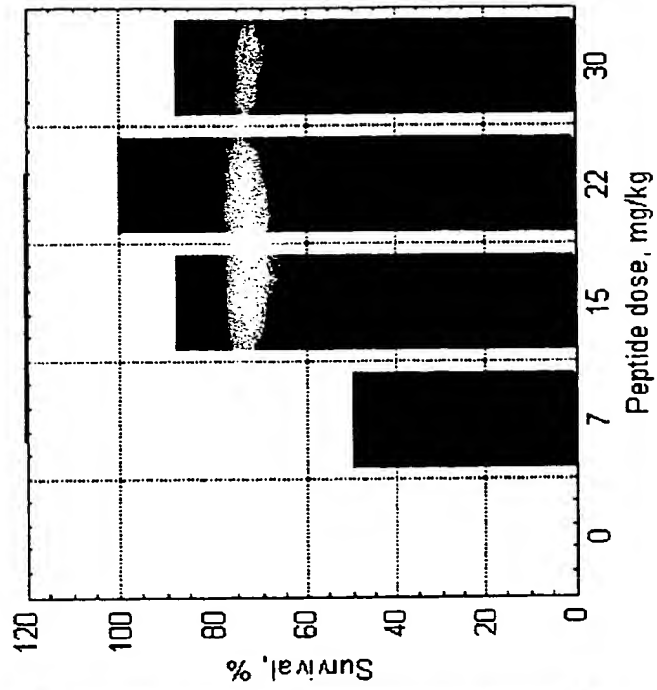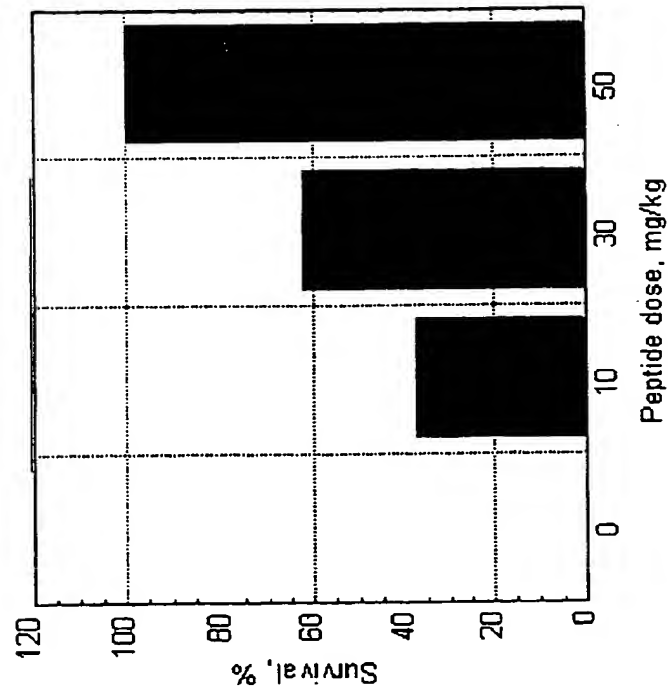
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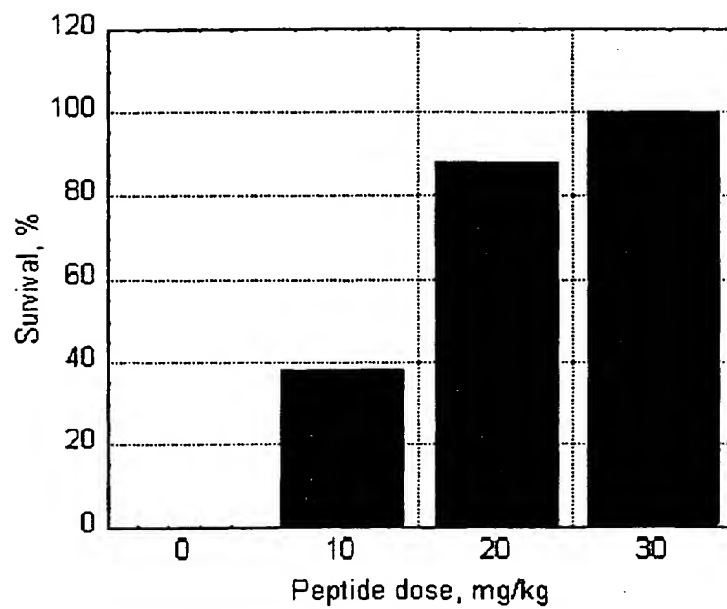
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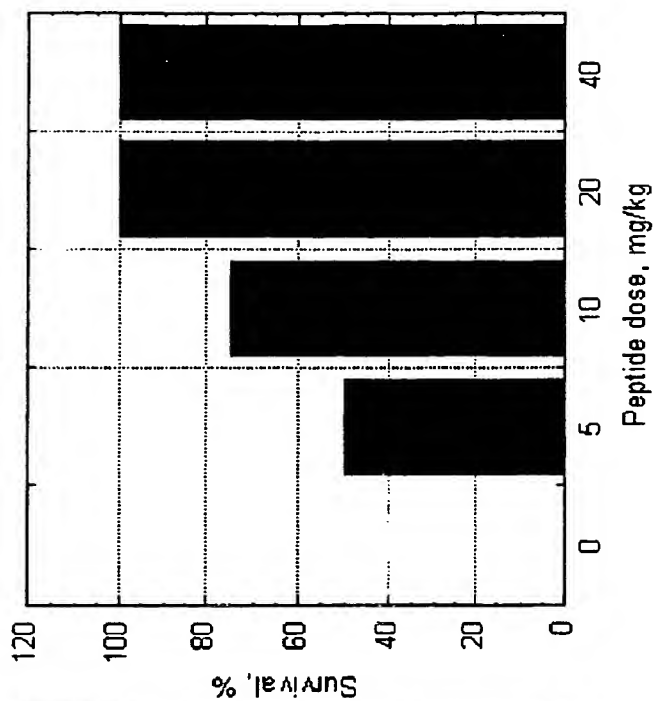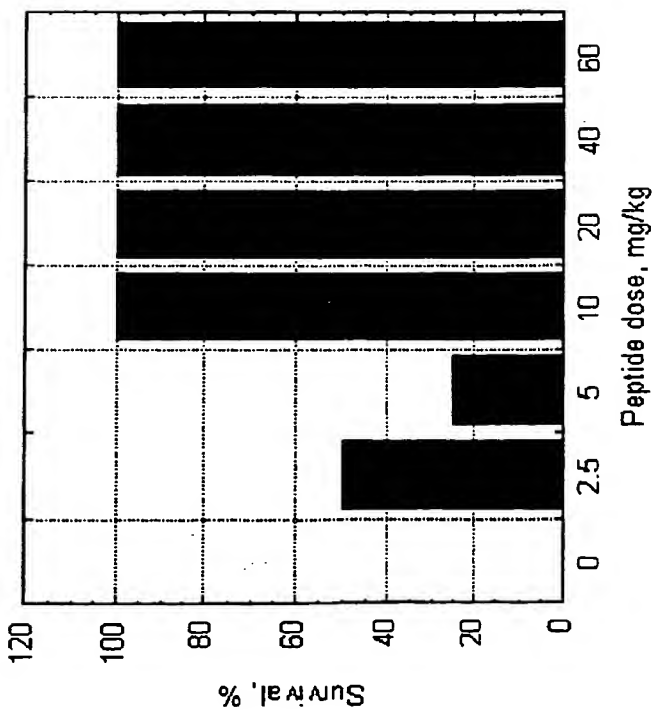
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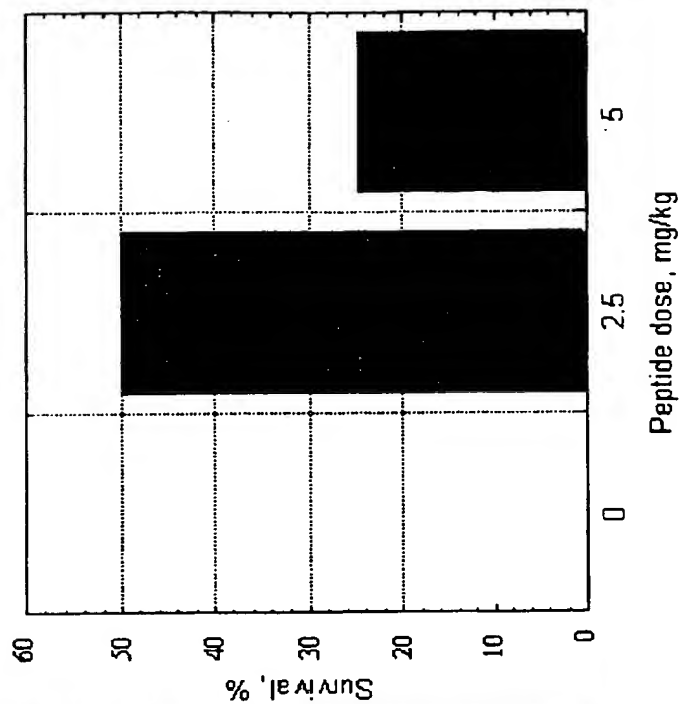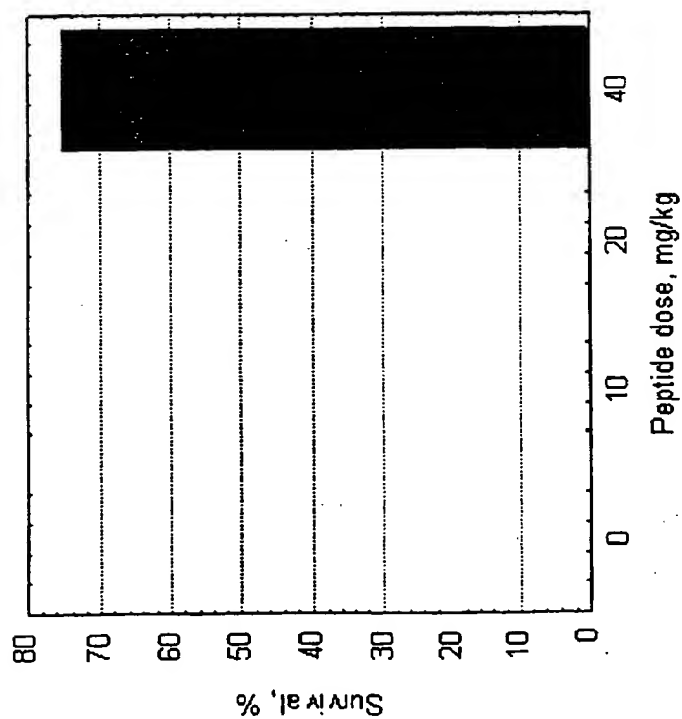
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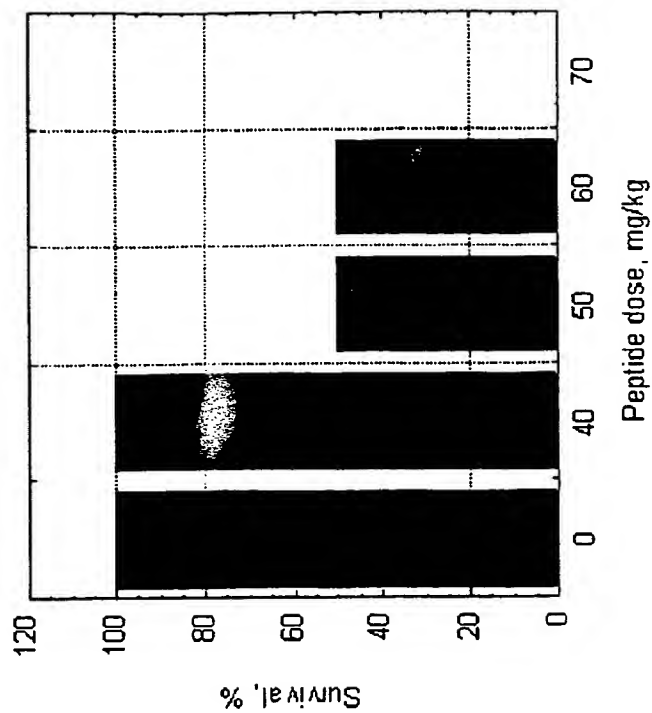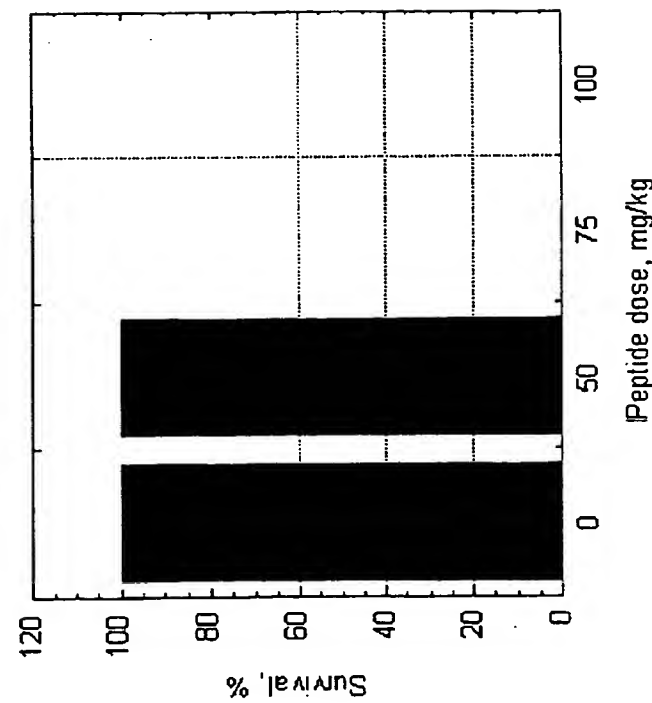
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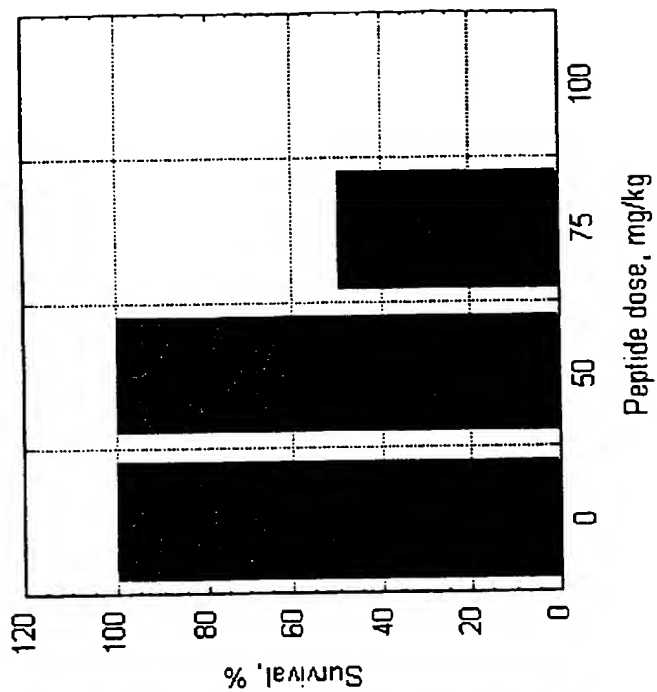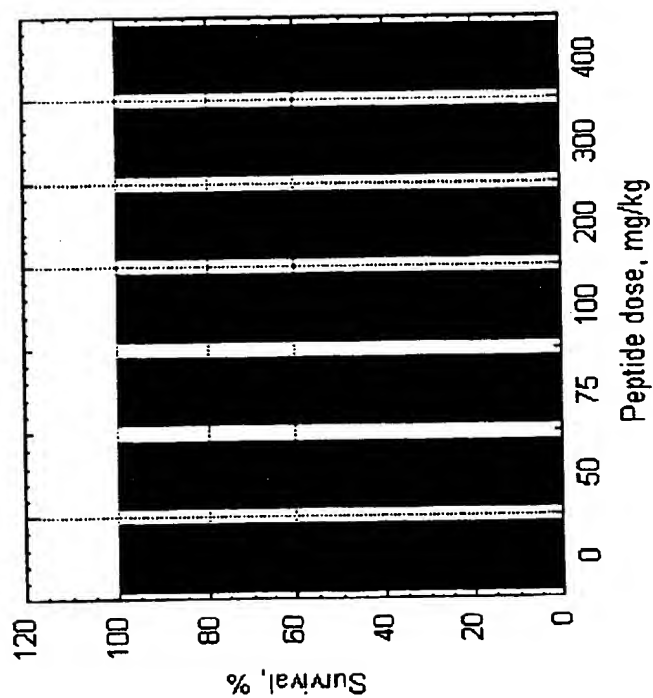
Fig. 30B

Fig. 30A

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5   Ghadiri, M.R.
         Wilcoxen, K.
         Fernandez-Lopez, S.
         Kim, H.

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<221> SITE
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   <222> 4, 6, 8
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 5  1               5


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20 <222> 4, 6, 8
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    <400> 62
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25 1               5


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    <221> SITE
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<221> SITE
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25 1   5

<210> 67
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    1               5


   <210> 68
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<223> Xaa = D-Trp.

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<210> 70
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<221> SITE
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25 1   5

<210> 71
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<221> SITE
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<210> 72
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<221> SITE
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<210> 73
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<221> SITE
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   <223> Xaa = D-His.


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25 1                    5


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    <223> Xaa = D-Lys.


 25 <221> SITE
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    <223> Xaa = D-Leu.


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     1                 5


    <210> 77
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   <221> SITE
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    1                   5


15 <210> 78
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<221> SITE
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<221> SITE
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   <221> SITE
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   <221> SITE
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30 1                 5


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  <223> Xaa = D-Leu.


25 <400> 86
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   1             5


  <210> 87
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5 1   5

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35 <210> 98
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 5 <221> SITE
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<221> SITE
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<221> SITE
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<210> 114
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(51) **International Patent Classification**[7]: C07K 7/64

(21) **International Application Number:**
PCT/US2002/014329

(22) **International Filing Date:** 6 May 2002 (06.05.2002)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
60/288,990 4 May 2001 (04.05.2001) US

(71) **Applicant** *(for all designated States except US)*: **THE SCRIPPS RESEARCH INSTITUTE** [US/US]; 10550 North Torrey Pines Road, La jolla, CA 92037 (US).

(71) **Applicants and**
(72) **Inventors: GHADIRI, M., Reza** [US/US]; 5133 Seagrove Court, San Diego, CA 92130 (US). **KIM, Hui-Sun** [US/US]; 1321 Everett Street, El Cerrito, CA 94530 (US). **FERNANDEZ-LOPEZ, Sara** [US/US]; 3945 Alma Street, Palo Alto, CA 94306 (US). **WILCOXEN, Keith** [US/US]; 730 Pennsylvania Avenue, San Diego, CA 92103 (US).

(74) **Agents: STEFFEY, Charles, E.** et al.; Schwegman, Lundberg, Woessner & Kluth, P.O. Box 2938, Minneapolis, MN 55402 (US).

(81) **Designated States** *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) **Date of publication of the international search report:**
29 January 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** ANTI-MICROBIAL PEPTIDES AND COMPOSITIONS

(57) **Abstract:** The present invention provides novel anti-microbial agents and compositions that include cyclic peptides having an amino sequence of alternating D-and L-α-amino acids. Alternatively, the cyclic peptides are made from β-amino acids.

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :   C07K 7/64

US CL   :   514/9, 11, 13, 16; 530/317, 300

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/9, 11, 13, 16; 530/317, 300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/10535 A1 (THE SCRIPPS RESEARCH INSTITUTE) 20 April 1995 (20.04.1995), pages 3-5, Figs 9-14 and 16.	134, 139, 144-147, 149-153, 156-162, 165 and 166
X — X	JOHNSON et al., Cyclic Peptides As Building Blocks for Extended Porous Solids, BOOK of Abstracts, 212th ACS National Meeting, August 25-29, 1996, MEDI-179, see abstract.	134, 139, 144-147, 149-152, 156-162, 165 and 166
X, P — X,P	FERNANDEZ-LOPEZ, et al., Antibacterial Agents based on the Cyclic D,L-alpha-peptide Architecture, Nature, 26 July 2001, Vol. 412, pages 452-455, see whole document.	1-4,6-39,41,6? 55-75,77-82,5--96,98,134,'8é-146,148-1?. ~.168,170,171,1 7,1 78 and 1.
X — X	GHADLRI, et al., SElf-assembling Organic Nanotubes Based on a Cyclic Peptide Architecture, Nature, 25 November 1993, Vol., 366, pages 324-327, see whoole document.	134, 139, 14-- '?, 149-152 and :.--162

☒ Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 September 2003 (01.09.2003)	17 NOV 2003
Name and mailing address of the ISA/US 　　Mail Stop PCT, Attn: ISA/US 　　Commissioner for Patents 　　P.O. Box 1450 　　Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Chih-Min Kam　　_Janice Ford_ Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAPAPORT, et al., Crystalline Cyclic Peptide Nanotubes at Interfaces, J. Am. Chem. Soc. 1999, Vol. 121, pages 1186-1191, see page 1187.	134, 139, 144, 149, 150 and 158-162

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/14329

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-41, 63-98, 134-171 and 175-180 (in part), SEQ ID NO:8.

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This international Search has found 36 Inventions claimed in the international Application covered by the claims indicated below:

Groups 1-39, claims 1-41, 63-98, 134-171 and 175-180, in part drawn to a peptide having anti-microbial activity comprising a cyclic peptide having a sequence of 4-16 alternating D-amino and L-a amino acids such as SEQ ID Nos: 8, 9, 12, 17, 18, 26, 29, 47-52, 61, 63, 67, 68, 72-77, 84, 85, 87-89, 91-93, 100, 102, 107, 111, 112, 119, 125 and 139; a pharmaceutical composition for treating or preventing a microbial infection in an animal comprising a pharmaceutically acceptable carrier and the cyclic peptide; and a method of treating a microbial infection in an animal comprising administering the cyclic peptide. For group 1, the claims will be searched to extent that they read upon SEQ ID NO:8; if group 2 were elected, the claims will be searched to extent that they read upon SEQ ID NO:9.

Group 40, claims 42-62 and 172-174, drawn to a pharmaceutical composition for treating or preventing a microbial infection in an animal comprising a pharmaceutically acceptable carrier and a cyclic peptide having a homochiral sequence of 3-10 b-amino acids, wherein at least one b-amino acid has at least one polar side chain.

Group 41, claims 99-119, drawn to a method for treating or preventing an infection caused by a target microbial organism in an animal comprising administering a cyclic peptide comprising a sequence of three to ten b-amino acids.

Group 42, claims 120-127, drawn to a method for identifying or evaluating a cyclic peptide with anti-microbial activity comprising contacting a target microbial organism with a test cyclic peptide, and determining whether the test cyclic peptide has anti-microbial activity, wherein the cyclic peptide comprises a sequence of 4-16 alternating D-amino and L-a amino acids, and wherein the contacting step is performed in vitro.

Group 43, claims 120-127, drawn to a method for identifying or evaluating a cyclic peptide with anti-microbial activity comprising contacting a target microbial organism with a test cyclic peptide, and determining whether the test cyclic peptide has anti-microbial activity, wherein the cyclic peptide comprises a sequence of three to ten b-amino acids, and wherein the contacting step is performed in vitro.

Group 44, claims 120-126 and 128, drawn to a method for identifying or evaluating a cyclic peptide with anti-microbial activity comprising contacting a target microbial organism with a test cyclic peptide, and determining whether the test cyclic peptide has anti-microbial activity, wherein the cyclic peptide comprises a sequence of 4-16 alternating D-amino and L-a amino acids, and wherein the contacting step is performed in vivo.

Group 45, claims 120-126 and 128, drawn to a method for identifying or evaluating a cyclic peptide with anti-microbial activity comprising contacting a target microbial organism with a test cyclic peptide, and determining whether the test cyclic peptide has anti-microbial activity, wherein the cyclic peptide comprises a sequence of three to ten b-amino acids, and wherein the contacting step is performed in vivo.

Group 46, claims 129-133, drawn to a method for identifying a cyclic peptide capable of selective association with a target biomolecule, comprising contacting a solution of cyclic peptides with the target microbial organism, and determining whether the peptide assemble into supramolecular structure thatselectively associates with the biomolecule, wherein the cyclic peptide comprises a sequence of 4-16 alternating D-amino and L-a amino acids.

This International Searching Authority considers that the international application does not comply with the requirements of unity invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups 1-46 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical features of Groups 1-39 are the specific peptide, the specific pharmaceutical composition and the specific method of treating a microbial infection in an animal comprising administering the cyclic peptide comprising a sequence of 4-16 alternating D-amino and L-a amino acids claimed therein. In contrast, the special technical features of Group 40 is the particular pharmaceutical composition for treating or preventing a microbial infection comprising a cyclic peptide comprising a sequence of three to ten b-amino acids claimed therein; the special technical features of Group 41 is the particular method of treating a microbial infection in an animal comprising administering the cyclic peptide comprising a sequence of three to ten b-amino acids claimed therein; the special technical features of Group 42 is the particular method for identifying a cyclic peptide comprising a sequence of 4-16 alternating D-amino and L-a amino acids with anti-microbial activity in

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vitro claimed therein; the special technical features of Group 43 is the particular method for identifying a cyclic peptide comprising a sequence of three to ten b-amino acids with anti-microbial activity in vitro claimed therein; the special technical features of Group 44 is the particular method for identifying a cyclic peptide comprising a sequence of 4-16 alternating D-amino and L-a amino acids with anti-microbial activity in vivo claimed therein; the special technical features of Group 45 is the particular method for identifying a cyclic peptide comprising a sequence of three to ten b-amino acids with anti-microbial activity in vivo claimed therein; the special technical features of Group 46 is the particular method for identifying a cyclic peptide comprising a sequence of 4-16 alternating D-amino and L-a amino acids capable of selective association with a target biomolecule claimed therein. Since the special technical feature of Group 1 is not present in the claims of Groups 2-46, and the special technical features of Groups 2-46 are not present in the claims of Group 1. Thus, unity of invention is lacking.

Continuation of B. FIELDS SEARCHED Item 3:
EAST search on USPAT, USPGPUB, DERWENT, EPO, JPO; STN search on MEDLINE, CAPLUS, BIOSIS, EMBASE, SCISEARCH, AGRICOLA; Amino Acid sequence search on SEQ ID NO:8. Search Term used: cyclic peptide, D-amino acid, L-amino acid, alternat?, antimicrobial, antibacterial, minimum inhibitory concentration, hemolysis, self-assembled, nanotube.

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